REMARKS

Claims 35-41, 47-50, 55-61, 67-69, 75-78, 80-82 and 86-90 are pending. No amendments to the claims have been made in this response and no new matter has been added. Applicants gratefully acknowledge the patentability of the pending claims under 35 U.S.C. §§ 102 and 103. The sole issue remaining is the enablement of certain claims directed to the treatment of cancer.[1]

The title of the present application has been amended to more closely reflect the pending claims.

I. The Rejection of Claims 35-41, 47-69 and 86-90 Under 35 U.S.C. § 112, First Paragraph

Claims 35-41, 47-69, 75-84 and 86-90 have been rejected under 35 U.S.C. § 112, first paragraph, for allegedly lacking enablement. In particular, while acknowledging that the specification is enabling for the inhibition of JNK, the Examiner has stated that the specification is not enabling for the treatment of cancer. Applicants respectfully disagree.

The Examiner has stated *inter alia* that the lack of guidance in the specification with regard to the actual treatment of cancer in a human makes practicing the claimed invention unpredictable. Applicants again respectfully point the Examiner to *In re Brana* which specifically addressed this issue. *In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995). Specifically, the Federal Circuit held that Title 35 does not demand that human testing occur within the confines of Patent and Trademark Office proceedings and that by requiring human *in vivo* data the Commissioner and the Board of Patent Appeals and Interferences confused the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption.[2] *In re Brana*, 51 F.3d at 1567. Using the Examiner's logic, no pharmaceutical case would be allowed until after FDA approval. Such is not the law.

The Examiner further stated that a method for treating cancer *in vivo* is highly speculative and that a greater amount of evidence is required to show operability in

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[1] Applicants note that pending claim 50 is also directed to conditions other than cancer, which do not appear to be objected to.

[2] Applicants note the Court's rational behind its holding: "Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer." *In re Brana*, 51 F.3d at 1567.

humans. In contrast, the Federal Circuit has stated that "treating cancer with chemical compounds does not suggest an inherently unbelievable undertaking or involve implausible scientific principles" as the Examiner seems to suggest. *Id.* Thus, Applicants respectfully submit that the treatment of cancer is not highly speculative.

The Examiner also notes that one would have to envisage and experiment with dosage, route of administration and duration of treatment. Applicants respectfully submit that experimentation by definition is the process of modifying and testing again. The correct inquiry is whether or not in the instant case the experimentation is undue. It is well-established that the fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *In re Certain Limited-Charge Cell Culture Microcarriers*, 221 U.S.P.Q. 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd sub nom., Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, (Fed. Cir. 1985); M.P.E.P. § 2164.01. Applicant respectfully submits that physicians routinely perform the tasks of determining dosage amounts and routes of administration regularly every day. Indeed, the Federal Circuit has held that a specification is enabling in part because those skilled in the art would know how to conduct a dose response study to determine the appropriate amounts to be used. *Merck & Co., Inc. v. Biocraft Laboratories, Inc.*, 874 F.2d 804, 809 (Fed. Cir. 1989). Thus, Applicant respectfully submits that it would not require undue experimentation to treat the claimed diseases with the claimed compounds, which have been shown to be JNK inhibitors.

Accordingly, Applicants respectfully submit that in the absence of some reason, other than the conclusory statement that treating cancer *in vivo* is highly speculative, the present specification must be taken as in compliance with the enabling requirement of 35 U.S.C. § 112, first paragraph. *In re Marzocchi*, 439 F.2d 220, 223 (C.C.P.A. 1971).

Nevertheless, Applicants submit herewith Force *et al.*, *Circulation 109(10)*:1196-1205 (2004) ("Force") and Manning *et al.*, *Nature 2*:554-565 (2003) ("Manning"), peer-reviewed publications which document the established correlation between *in vitro* kinase inhibition assays and *in vivo* use and, accordingly, rebut any doubt one skilled in the art might have regarding the treatment of cancer *in vivo* with a kinase inhibitor, such as compounds of the present invention. In particular, Applicants believe that the disclosure of Force and Manning satisfy the Examiner's concern regarding a correlation between *in vitro* tests and the use of the active agents *in vivo*.

Force states that the pathophysiological dysfunction of protein kinase signaling pathways underlies the molecular basis of many cancers (*see* Abstract) and even predicts that in the future, cancers will be defined not only by tumor type and stage but also by the protein kinase activity profile (*i.e.*, which kinases are dysregulated) (*see* page 1197, first column, lines 34-38). Thus, it is clear that those skilled in the art recognize the nexus between kinase activity and cancer, as well as various other disorders.

Indeed, the extensive list of small molecule kinase inhibitors set forth at pages 1198-1199 of Force which have either successfully completed or are currently in human clinical trials illustrates the belief by those skilled in the art that kinase inhibitors are effective for the treatment of cancer. Applicants would like to point out that the last agent set forth in the table at page 1198 of Force (*i.e.*, CC401) is an indazole compound which falls within the scope of the present method of use claims. Three small Phase I clinical trials directed in part to determine the optimal biological does of CC401 have been completed and a fourth is currently scheduled. The completed trials have included both healthy volunteers and patients with acute myeloid leukemia.

Manning is a review article which discusses the evidence supporting the application of JNK inhibitors to treat inflammatory, vascular, neurodegenerative, metabolic and oncological disease in humans (*see* page 554, last line of abstract). Regarding cancer, Manning summarizes the data pointing to the link between JNK activity and a wide variety of cancers (*e.g.*, pancreas, lung, breast, colon and prostate) and suggests that JNK could play more than one role in tumour development (*see*, "Cancer" at pages 561-562).

Force and Manning are representative of the extensive literature which points to kinases, especially JNK, as important therapeutic targets for the treatment of cancer and various other disorders.

In summary, Applicants submit that the disclosure of the present application, in combination with what is known in the art regarding small molecule kinase inhibitors, satisfies the enablement requirement of 35 U.S.C. § 112, first paragraph. Specifically, the present application provides small molecule kinase inhibitors (which the Examiner has acknowledged are enabled for inhibiting JNK) and the literature demonstrates not only that kinases are accepted therapeutic targets for a number of diverse diseases (*e.g.*, cancer, diabetes, inflammation, stroke, Crohn's disease and neurodegeneration), but that a significant number of clinical trials directed to the use of small molecule kinase inhibitors

are in fact ongoing. Thus, it is within the means of those skilled in the art to practice the present claims (such as treating cancer with JNK inhibitors provided by the present application) without undue experimentation.

For the reasons set forth above, Applicants believe that the specification enables the remaining pending method of treatment claims and, accordingly, that the rejection under 35 U.S.C. § 112, first paragraph, cannot stand and must be withdrawn.

II. Previously Submitted Form PTO-1449 Listing References AA-BS

Applicants respectfully request that references AA-BS set forth in the Form PTO-1449 titled, "List of References Cited by Applicant," that was filed concurrently with the above-identified application on September 26, 2003, be made of record in the file history of the above-identified application by initialing the Form PTO-1449 and returning to Applicants.

Conclusion

Applicants respectfully request that the above remarks be entered in the present application file. No fee is believed to be due in connection with this Response; however, in the event that any fee is due, please charge the required fee to Jones Day Deposit Account No. 50-3013.

Respectfully submitted,

Date: November 9, 2005

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Enclosures

Inhibitors of Protein Kinase Signaling Pathways

Emerging Therapies for Cardiovascular Disease

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Abstract—Protein kinases are enzymes that covalently modify proteins by attaching phosphate groups (from ATP) to serine, threonine, and/or tyrosine residues. In so doing, the functional properties of the protein kinase's substrates are modified. Protein kinases transduce signals from the cell membrane into the interior of the cell. Such signals include not only those arising from ligand–receptor interactions but also environmental perturbations such as when the membrane undergoes mechanical deformation (ie, cell stretch or shear stress). Ultimately, the activation of signaling pathways that use protein kinases often culminates in the reprogramming of gene expression through the direct regulation of transcription factors or through the regulation of mRNA stability or protein translation. Protein kinases regulate most aspects of normal cellular function. The pathophysiological dysfunction of protein kinase signaling pathways underlies the molecular basis of many cancers and of several manifestations of cardiovascular disease, such as hypertrophy and other types of left ventricular remodeling, ischemia/reperfusion injury, angiogenesis, and atherogenesis. Given their roles in such a wide variety of disease states, protein kinases are rapidly becoming extremely attractive targets for drug discovery, probably second only to heterotrimeric G protein–coupled receptors (eg, angiotensin II). Here, we will review the reasons for this explosion in interest in inhibitors of protein kinases and will describe the process of identifying novel drugs directed against kinases. We will specifically focus on disease states for which drug development has proceeded to the point of clinical or advanced preclinical studies. (***Circulation.* 2004;109:1196-1205.**)

Key Words: drugs ■ kinases ■ pharmacology ■ inhibitors

A consensus is emerging that protein kinase modulators will be effective treatments for a variety of diseases.[1] However, protein kinases were initially thought to be unsuitable drug targets, in large part because of what was perceived to be an unfavorably high degree of structural conservation within key domains of all protein kinases. Because binding of ATP to kinases is essential for kinase activity and properties of the protein kinase ATP-binding pocket were well understood, agents targeting the ATP pocket were the logical first choice for drug development. However, the structural conservation of protein kinase ATP binding sites and the presence of more than 500 protein kinases in the human genome[2] led to the belief that highly selective small-molecule protein kinase inhibitors targeting the ATP pocket would be difficult to generate. As will be discussed below, the development and characterization of inhibitors of the p38 mitogen-activated protein kinases (MAPKs) indicated that this initial belief was misguided. A second argument against targeting protein kinases for drug development was the observation that modulation of a protein kinase could in one system prove beneficial, while proving deleterious in another. As an extreme example of this, inhibiting a protein kinase required for triggering programmed cell death could reduce ischemia-induced cell death in terminally differentiated cardiomyocytes but might also favor tumor promotion in other organs or cell types. Finally, toxicity with long-term use was a concern. Thus, inhibiting a protein kinase that is dysregulated in one organ in a particular disease state may prove harmful to other systems in which that same protein kinase is not dysregulated but instead serves essential functions. For example, inhibiting the cell-surface HER2 tyrosine kinase receptor with the monoclonal antibody trastuzumab (Herceptin, Genentech) in patients with breast cancers overexpressing that receptor has produced strikingly beneficial results, but it has come at the expense of severe cardiac dysfunction in some women receiving the therapy, suggesting a critical role for this receptor in cardiomyocyte survival.[3]

All of the above concerns being noted, the "proof of principle" of the tremendous therapeutic potential of small-molecule inhibitors of protein kinases came with the discov-

From the Molecular Cardiology Research Institute, Tufts-New England Medical Center and Tufts University School of Medicine, Boston, Mass (T.F., J.M.K.); Vertex Pharmaceuticals, Inc, Cambridge, Mass (K.K., M.N.); and the Department of Biomedical Sciences, University of Rhode Island, Kingston (K.P.).

Drs Namchuck and Kuida are employees of and Dr Force receives financial support for his laboratory from Vertex Pharmaceuticals, Inc, which produces small-molecule inhibitors of protein kinases, are the subject of this article.

Additional material may be found in the Data Supplement with the online-only version of this article at http://www.circulationaha.org.

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Circulation is available at http://www.circulationaha.org DOI: 10.1161/01.CIR.0000118538.21306.A9

ery of imatinib mesylate (Gleevec, STI-571, Novartis), an ATP-competitive small-molecule inhibitor of the tumorigenic fusion protein Bcr-Abl (reviewed by Barnes and Melo[4]) (Table; Figure 1). c-Abl is a nuclear protein tyrosine kinase the biological function of which is unclear (although it may function in sensing the integrity of the genome and promoting programmed cell death). Bcr is a multifunctional cytosolic polypeptide that may play a role in regulating activity of the Rho subfamily of small G proteins. The fusion of Bcr and Abl to produce Bcr-Abl arises from the chromosomal translocation that creates the Philadelphia chromosome. Unlike c-Abl, Bcr-Abl is both cytosolic and nuclear, and because it forms homodimers that cross-phosphorylate and activate one another, Bcr-Abl manifests constitutively active and inappropriately directed Tyr kinase activity. Bcr-Abl is causal in chronic myelogenous leukemia, and treatment with imatinib has been able to induce complete remissions, at least in the early stages of the disease.[4]

Indeed, the cancer field has led the way in spurring on drug development directed both at protein kinases that, like Bcr-Abl, are activated by mutations and lead directly to growth deregulation and at "permissive" protein kinases that, while otherwise normal themselves, serve as essential effectors for mutant, deregulated gene products. The protein kinases MAPK ERK kinase (MEK)1/2, which activate the extracellular signal–regulated kinase (ERK) family of MAPKs (Figure 2), and the mammalian target of rapamycin (mTOR) are 2 such permissive kinases that play roles in cell cycle progression. Inhibitors of these kinases (U0126 and PD184352 [Figure 1] and rapamycin/sirolimus, respectively) are in clinical trials for the treatment of a variety of tumors (Table). In addition, rapamycin/sirolimus is currently used with dramatic success as an immunosuppressant and an inhibitor of in-stent restenosis.[5] Early successes with agents targeting protein kinases have led to the logical conclusion that in the future, cancers will be defined not only by tumor type and stage but also by the protein kinase activity profile (ie, which kinases are dysregulated).[6] It is likely that the same will be true for complex disease states of the cardiovascular system.

Developing an Inhibitor

A major issue in drug development is the identification of appropriate targets for therapeutic intervention. To identify a protein kinase as a putative therapeutic target, it is not sufficient simply to know whether it is activated (or inhibited) in a specific disease state, because dysregulation can be an irrelevant consequence of the disease rather than a key contributing factor to disease pathology. At the very least, clear genetic or physiological/cell biological data are needed that implicate a protein kinase as an attractive target.

Once a kinase is validated as a potential target for drug development, screening of chemical libraries is performed to identify possible inhibitors. Many large pharmaceutical companies possess enormous chemical libraries consisting of hundreds of thousands of synthetic compounds. The identification of one or more of these as a candidate inhibitor requires a process called high-throughput screening (HTS). (For the interested reader, a more detailed description of the process of HTS is available on-line and in Reference 7.) A good, robust, and reliable HTS assay can be used to screen >100 000 small molecules in a day. Typical "hit rates" for an unbiased screen might be only 0.1% to 0.3%; therefore, various strategies have been devised to improve hit rates by focusing the screen. Focusing of the library of compounds can be based on the actual crystal structure of the ATP-binding pocket of the kinase or a family member if known (structure-based library design) or on the structure of compounds already known to bind to the ATP pocket if available (ligand-based library design). These virtual screening or molecular modeling approaches to screen more targeted libraries not only can improve the hit rate but also may reduce the duration and expense of primary screens.[7]

Binding of ATP to a protein kinase is essential for the kinase's phosphotransferase activity, and thus, the ATP-binding pocket is the "target" of most inhibitor screens. As was noted above, this idea initially seemed counterintuitive, given the structural conservation of protein kinase ATP-binding sites.[8] However, there is, in fact, enough structural diversity in these sites[8] to predict that selective ATP-competitive inhibitors can be identified. Indeed, contrary to initial concerns, screens of unbiased compound libraries have identified several ATP competitors that function as relatively selective inhibitors.[9,10]

For a protein kinase inhibitor to have a chance of clinical efficacy, it must bind to the target kinase with an extremely high affinity: several orders of magnitude higher than that of ATP, because the inhibitor will be present in concentrations typically in the mid to high nanomolar range, whereas the intracellular concentration of ATP is millimolar. This suggests that any initial "hits" from an HTS will most likely benefit from optimization to improve potency and selectivity. The efficiency of the optimization process is greatly augmented by the abundant x-ray crystallographic information available for kinase families. Thus, the structure–activity relationship of any compound can be correlated with specific molecular interactions of the compound with the kinase active site, and in this way, the structure of the inhibitor can be optimized.[7] When no structure data exist for a specific kinase, knowledge of the structure of another member of the family can often be used to create binding models from which optimized compounds can be synthesized.[7]

The need for an extremely high binding affinity of an inhibitor to the ATP pocket and the relative similarities of ATP pockets across protein kinase families suggest that it may be beneficial to examine protein kinases for determinants in addition to the ATP pocket that might confer additional specificity. Here, the MAPKs provide an excellent example. The ability of different MAPK groups to interact with and then phosphorylate selective intracellular protein substrates is conferred by a specific substrate docking site of the MAPKs, the common docking (CD) domain, that is quite distal from the ATP binding site.[11] The CD motifs of MAPKs bind complementary sites on the corresponding MAPK substrates (and on MAPK regulators) (eg, MEK1 binding to ERK-1 [Figure 2] is mediated by the CD domain). Although there is substantial sequence conservation among MAPK CD domains, the sequence divergence is sufficient to enable

Selected Inhibitors of Protein Kinases in Clinical Trials

Kinase Target	Agent	Trial (Disease)	Sponsor
Tyrosine kinases			
ABL (c-Kit, PDGFR)	Gleevec (STI-571)	Approved (CML)	Novartis
EGFR	ZD1839 (Iressa)	Approved (lung cancer)	AstraZeneca
	OSI-774	Phase III (cancer)	OSI/Roche/Genentech
	IMC-C225 (mAb)	Phase III (cancer)	ImClone
	ABX-EGF (mAb)	Phase II (cancer)	Abgenix
	MDX-447 (mAb)	Phase I (cancer)	Merck KgaA
	EMD 72000 (mAb)	Phase I (cancer)	Merck KgaA
	Genistein	Phase II (cancer)	NCI
	RH3 (mAb)	Phase II (cancer)	York Medical Bioscience Inc
EGFR, ERB2R	CI1033	Phase II (cancer)	Pfizer
	EKB569	Phase I (cancer)	Wyeth-Ayerst
	GW2016	Phase I (cancer)	GlaxoSmithKline
	PKI166	Phase I (cancer)	Novartis
VEGFR (PDGFR, FGFR)	SU6668	Phase I (cancer)	Pharmacia Corp
PDGFR (Flt-3)	CT53518	Phase I (cancer)	Millennium Pharmaceuticals
VEGFR	SU5416	Phase III (cancer)	Pharmacia Corp
	PTK787/ZK222584	Phase II (cancer)	Novartis/Schering-Plough
VEGFR (EGFR)	ZD6474	Phase II (cancer)	AstraZeneca
VEGFR (PDGFR)	SU011248	Phase II (cancer)	Sugen
NGFR, Trk	CEP-2583	Phase II (cancer)	Cephalon
HER-2/neu	17-AAG	Phase I (cancer)	Kosan
	Trastuzumab (mAb)	Approved (cancer)	Genetech
	2C4 (mAb)	Phase I (cancer)	Genetech
	CP-724,714	Phase I (cancer)	OSI Pharmaceuticals/Pfizer
	MDX-210 (mAb)	Phase I (cancer)	Novartis
Serine/threonine kinases			
PKC, c-Kit, PDGFR	PKC412	Phase II (cancer, retinopathy)	Novartis
PKC	ISIS 3521	Phase III (cancer)	ISIS Pharmaceuticals
	CGP41251	Phase II (cancer)	Novartis
	UCN-01	Phase I/II (cancer)	Kyowa Hakko Kogyo
	Bryostatin-1	Phase I/II (cancer)	Biotek
PKC-β	Ly333531	Phase I (cancer)	Eli Lilly
		Phase II/III (diabetic neuropathy)	
CDKs	Flavopiridol	Phase II (cancer)	Aventis
	E7070	Phase I (cancer)	EISAI
	BMS-387032	Phase I (cancer)	Bristol-Myers Squibb
	CYC202	Phase I (cancer)	Cyclacel
MEK1/2	PD184352	Phase II (cancer)	Pfizer
	U-0126	Phase I (cancer)	Promega
MLK	CEP-1347	Phase II (neurodegeneration)	Cephalon
RAF	BAY43–9006	Phase II (cancer)	Onyx Pharmaceuticals/Bayer
	ISIS5132	Phase II (cancer)	Isis pharmaceuticals
	L-779,450	Phase II (cancer)	Merck
Ras	ISIS2503	Phase II (cancer)	Isis pharmaceuticals
	SCH66336	Phase II (cancer)	Schering-Plough
	BMS214662	Phase I (cancer)	Bristol-Myers Squibb
	R115777	Phase I/II (cancer)	Johnson & Johnson
mTOR	CCI779	Phase II (cancer)	Wyeth-Ayerst
	RAD001	Phase I (cancer)	Novartis
		Phase II/II (immunosuppressant)	
	Rapamycin	Approved (immunosuppressant)	Wyeth-Ayerst
p38-MAPK	VX702	Phase II (inflammation; ACS)	Vertex Pharmaceuticals
	BIRB796	Phase III (inflammation; RA; Crohn's)	Boehringer Ingelheim
	SCIO-323	Phase I (RA; stroke; diabetes)	Scios, Inc
	SCIO-469	Phase II (RA; Crohn's)	Scios, Inc
PDK1	UCN-01	Phase I/II (cancer)	Kyowa Hakko Kogyo
JNK1–3	CC401	Phase I	Celgene

VEGFR indicates vascular endothelial growth factor receptor; PDGFR, PDGF receptor; FGFR, fibroblast growth factor receptor; CML, chronic myelogenous leukemia; RA, rheumatoid arthritis; and ACS, acute coronary syndromes. Inhibitors are of two types, monoclonal antibodies (mAbs), which are directed at the extracellular domain of various receptor tyrosine kinases, and small-molecule inhibitors.

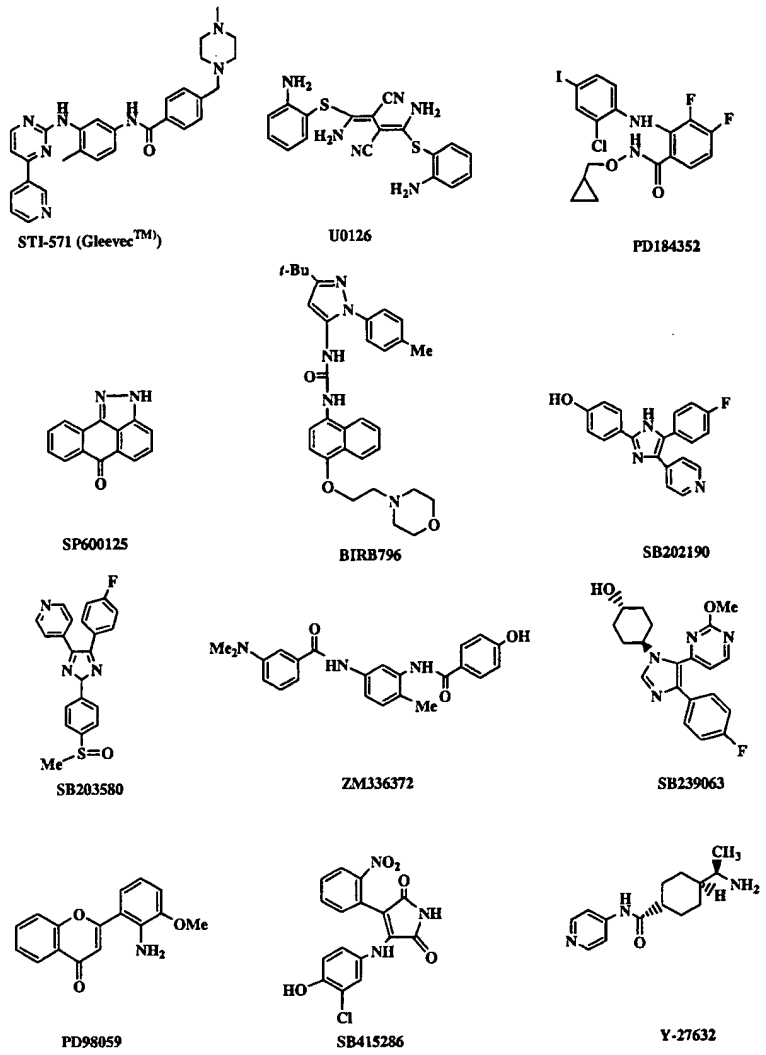

Figure 1. Chemical structures of several small-molecule protein kinase inhibitors referred to in text. These can be divided into inhibitors that are ATP-competitive, including phenylamino pyrimidines (eg, STI-571), pyridinylimidazoles (SB202190, SB203580, and SB239063), anthrapyrazolones (SP600125), and maleimides (SB415286), and those that are non–ATP-competitive (MEK1/2 inhibitors, U0126, PD184352, and PD98059, which maintain kinases in an inactive state by preventing their phosphorylation by upstream activating kinases such as Raf). BIRB796, a pyrazole urea, is both noncompetitive and competitive (see text).

exquisite MAPK specificity. Of note, the CD domains are quite small (≤18 amino acids), contain key acidic residues, and reside on an exposed surface in the MAPK structure, suggesting that these domains could be ideal targets for drug design.[11]

The use of determinants in addition to the ATP pocket combined with optimization based on crystal structure was recently used to optimize the design of a p38-MAPK inhibitor. Crystallography demonstrated that this inhibitor did not target the ATP binding pocket but rather targeted a novel site in the kinase active site that is exposed after a large conformational change that accompanies binding of the inhibitor.[12] Crystallography allowed the compound to be modified to optimize binding to the novel site and also to establish binding in the ATP pocket. This gives the final compound, BIRB796 (Figures 1 and 3), which is currently in clinical trials for various inflammatory disorders (Table), a high degree of potency and selectivity.

Another approach to inhibit MAPK signaling that might reduce toxicity would be to target upstream activators of the MAPKs rather than the MAPKs.[13] For example, c-Jun N-terminal kinase (JNKs) are activated by at least 12 different MAPK kinase kinases (MAPKKKs) and 2 MAPK kinases (MAPKKs: see legend to Figure 2 for terminology). Because specific MAPKKKs and MAPKKs transduce the activation of JNKs in response to specific stimuli[14] (eg, MAPKK7 but not MAPKK4 is necessary for JNK activation by tumor necrosis factor [TNF]-α), one could potentially target MAPKK7 specifically with an inhibitor in patients with inflammatory disorders. This would leave JNK activation by other stimuli acting via MAPKK4, and essential cellular functions regulated by JNKs, at least partially intact.

Potency and Selectivity

Potency and selectivity are critical issues for the eventual effectiveness and safety of any drug. Potency is expressed as the enzymatic IC_{50} (concentration of drug that inhibits enzyme activity by 50%). However, reported IC_{50}s must be interpreted with caution, because the IC_{50} determined for an ATP-competitive inhibitor will vary depending on the concentration of ATP used in the assay and on the K_m (the affinity of the kinase for ATP).[10] This has been a source of significant confusion in the literature. For example, results from assays of the widely used anthrapyrazolone JNK inhib-

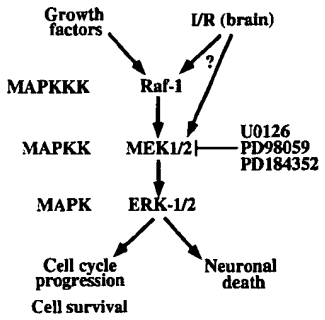

Figure 2. ERK cascade. All MAPKs described to date are part of a 3-tiered cascade whereby MAPKs, in this case, ERKs, are activated by upstream kinases (MAPKKs, in this case MEK1/2), which, in turn, are activated by a MAPKKK (in this case Raf-1). Growth factor–induced activation of pathway often leads to cell cycle progression and, in some cases, activation of survival pathways. I/R in brain also leads to ERK activation, but in this case it is deleterious, leading to neuronal death. It is not clear whether Raf-1 is the MAPKKK involved in I/R-induced activation of ERKs in brain. MEK inhibitors discussed in text are shown.

itor SP600125 (Figure 1) with 20 μmol/L ATP initially suggested that SP600125 was a very potent inhibitor with a low IC_{50}. However, the results of studies that used assays with more "physiological" concentrations of ATP (100 μmol/L) recently demonstrated that SP600125 was, in fact, a relatively weak (and also nonselective) inhibitor with a high IC_{50}.[10]

Selectivity is a second key consideration in the design of kinase inhibitors. Compounds are "profiled" for their selectivity against panels of kinases (often 30 or more) to determine which targets, aside from the intended one, are being affected. These panels are chosen in a variety of ways but often include specific kinases that one does not want the drug to inhibit and/or a selection of kinases with a great deal of structural diversity at the active site (to broadly screen for nonspecific inhibition). Relative IC_{50}s of the drug for the target kinase versus the others in the panel are then determined. Again, the concentration of ATP used in the assay is critical to allow an accurate comparison to be made. One approach to allow interpretation of relative IC_{50}s for an inhibitor between enzymes is to customize the assay conditions for the ATP affinity for each kinase in the screening panel (eg, fix the ATP concentration at the K_m for each kinase). Alternatively, others recommend using concentrations of ATP, ≥100 μmol/L, that are well above the K_m for all of the kinases in the panel.[10]

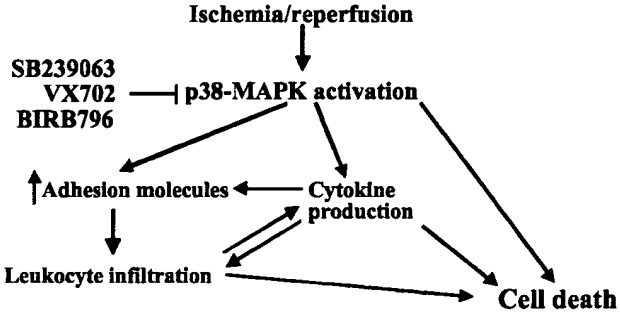

Figure 3. Mechanisms of p38-MAPK–induced cardiomyocyte death. I/R activates p38-MAPK, leading to both cytokine (and chemokine) production and upregulation of adhesion molecules on endothelial cells. This leads to leukocyte infiltration into ischemic region. Certain cytokines (eg, TNF-α) are directly cytotoxic to cardiomyocytes. In addition, p38-MAPK probably also directly activates cell death pathways in ischemic cardiomyocytes (ie, cytokine- and leukocyte-independent effects on cell death). p38-MAPK inhibitors discussed in text are shown. Of note, JNKs also act to stabilize cytokine mRNA and, in addition, activate intrinsic cell death pathway by inducing release of cytochrome c from mitochondria.[36] This suggests that JNK inhibitors may also be protective against I/R injury (see text).

What is an acceptable level of selectivity? There is no consensus, but in general, the goal is an IC_{50} that is at least 100-fold lower for the target kinase. However, this may vary depending on the indication, and in some cases, one might tolerate (or even prefer) agents that are not entirely selective. For example, in cancer, one might tolerate inhibition of kinases that positively regulate the cell cycle (cyclin-dependent kinases, Cdks) or that are antiapoptotic (eg, Akt) by a drug targeting Bcr-Abl, because antitumor activity might be greater. However, because of enhanced toxicity, one would not tolerate inhibition of Cdks or Akt by a drug targeting p38-MAPK for inflammatory diseases. Similarly, lack of selectivity for drugs that will be used short-term only might not be a major problem.

Once selectivity is determined in kinase assays in vitro, the selectivity profile is then determined in a cellular system. Given that cellular ATP concentrations are typically in the millimolar range, an upward shift in the cellular IC_{50} versus the enzymatic IC_{50} (performed at 100 μmol/L or less) is often observed. The magnitude of this shift is dictated by a number of factors, including the ATP K_m for the target enzyme, the cellular permeability of the drug, and the amount of inhibition of the target kinase required to elicit the cellular response being monitored (eg, 20% inhibition of a particular kinase may be sufficient to lead to complete inhibition of a biological response). An effective general counterscreening strategy is to obtain enzymatic IC_{50} values for an extensive panel of biochemical kinase assays, then assess the cellular consequences of the observed inhibition pattern in cellular readouts biased to respond to inhibition of the signaling pathways represented in the enzymatic panel. If a drug with borderline selectivity in enzymatic assays has excellent characteristics in the cell-based assays (good inhibition of the target pathway, limited inhibition of other pathways, and no toxicity), the borderline enzymatic selectivity may be deemed adequate.

Finally, although the IC_{50} and selectivity studies (determined in assays in vitro) usually predict activity in the cell, this is not always the case. Thus, a compound with apparent high activity and specificity in vitro may display markedly different and even unexpectedly nonspecific activity in vivo. For example, the pyridinyl imidazoles SB203580 and SB202190 (Figure 1), which inhibit p38 MAPKs, are remarkably specific when assayed in vitro for inhibition of a variety of protein kinases.[9] The basis for this specificity was revealed in the crystal structure of p38α complexed with SB203580. To accommodate a fluorophenyl moiety present in the SB203580 structure, the amino acid at position 106 of the

kinase must be no larger than Thr.[15] c-Raf, a protein kinase that activates the ERKs, is downstream of many growth factor receptors and plays a role in inducing cell-cycle progression (Figure 2), has a Thr (Thr321) at a site corresponding to Thr106 of p38α. Not unexpectedly, therefore, c-Raf is inhibited by SB203580 and SB202092 in vitro, albeit at concentrations at least an order of magnitude higher than that needed to inhibit p38α.[13,14] However, in cell-based assays, the Raf–Mek-ERK pathway is not inhibited by SB203580 or SB202092. Surprisingly, SB203580 and SB202092 trigger a striking activation of c-Raf in vivo.[16] Similarly, ZM336372 (Figure 1), a novel phenylamido derivative, is an in vitro Raf (and p38-MAPK) inhibitor but is a potent activator of c-Raf in intact cells. The basis for these paradoxical findings is unknown, but they are indicative of the fact that assertions as to the specificity of a compound in vitro require rigorous and comprehensive testing in cellular and whole-animal systems.

To the Bedside

The Table is a listing of most of the protein kinase inhibitors currently in clinical trials and the diseases targeted. As can be seen, most are cancer trials, but there is a trend toward targeting protein kinases for the treatment of a number of chronic conditions other than cancer, including inflammatory and cardiovascular diseases. Indeed, several of the agents listed in the Table have strong preclinical data suggesting that they may be efficacious in the therapy of patients with a variety of cardiovascular diseases. The list of potential protein kinase targets for cardiovascular therapies is extensive. However, rather than a summary of disease states and protein kinases possibly involved (an excellent review taking this approach for heart failure was recently published[17]), we will discuss a few disease states for which inhibitors exist that are either already in the earliest stages of clinical trials or are in the late stages of preclinical development. These examples, we hope, will illustrate that what was once perceived to be impractical now seems reasonable and attainable.

Acute Coronary Syndromes

Two families of stress-activated MAPKs, the JNKs and p38-MAPKs, are activated by ischemia/reperfusion (I/R),[14,18] and there is some indication that inhibition of either the JNKs or p38s might prove beneficial for treating acute coronary syndromes (ACS). However, validating these MAPKs as targets in ACS, that is, whether activation of the kinases is beneficial or detrimental, has been difficult.[19] This is because of the lack of good genetic models (ie, mice deleted for the gene) and, until recently for p38, good inhibitors with which to address the question in vivo. Two members of the p38 MAPK family, p38α and p38β, are activated by ischemia. The first effective inhibitor of p38α/β was discovered by Lee and coworkers[20] at SKF in a broad-based screen for "cytokine-suppressive antiinflammatory drugs" based on their ability to inhibit endotoxin-induced cytokine production by macrophages in culture. The target of this drug was later identified to be the p38s. Because it seems clear that the first wave of drugs targeting kinase pathways to be used in patients will be dominated by p38-MAPK inhibitors, we will describe these kinases and the mechanisms by which the inhibitors work in some detail.

Preventing the release of inflammatory cytokines and chemokines represents a potentially promising approach to treating ACS (Figure 3) and, possibly, the development and progression of atherosclerotic plaques. Indeed, a p38 inhibitor, VX702, is currently in a phase II clinical trial in patients presenting with ACS. The half-life of the mRNA for many cytokines (and growth factors) is extremely short, allowing for rapid downregulation of expression when the inciting stimulus is removed. This short half-life is largely a result of the presence of AU-rich elements (AREs, consisting of several copies of the sequence AUUUA) in the 3′-untranslated region of the mRNA.[21] ARE-binding proteins (ARE-BPs) bind to the AREs, and most ARE-BPs target mRNA for degradation. When activated, p38s phosphorylate ARE-BPs, inhibiting their activity.[21] The end result is p38-dependent stabilization of the cytokine mRNA, leading to increased production of the cytokine protein and activation of inflammatory cells and of endothelial cells, the latter leading to upregulation of adhesion molecules. Thus, p38 inhibitors block phosphorylation of the ARE-BPs, leading to degradation of the cytokine mRNAs, including those coding for TNF-α, interleukin (IL)-1α/β, IL-6, IL-10, interferon (IFN)-γ, MIP1α/β, and IL-8. Although stabilization of cytokine mRNA has obviously been an important response to infection over millions of years of evolution, inappropriate activation of inflammatory responses has, over the past 100 years, become a significant factor driving the explosion in the prevalence of a number of chronic disease states.

Several companies have developed p38 inhibitors, and some of these have demonstrated efficacy in models of inflammatory diseases, including inflammatory arthritides and inflammatory bowel disease, as well as in endotoxemia.[22,23] Some of these inhibitors are currently in clinical trials for rheumatoid arthritis and Crohn's disease (Table). With the rationales that (1) ACSs, including myocardial infarction, had prominent inflammatory components and (2) p38 activation in ischemic tissue might, independent of effects on inflammatory responses, have detrimental effects on cardiomyocyte survival (see Reference 17 and references therein), the efficacy of these drugs was tested in animal models of acute myocardial infarction.

Early-generation p38 inhibitors, SB203580 and SB242710, reduced I/R-induced apoptosis and preserved cardiac function in a Langendorff-perfused rabbit heart model (reviewed in Reference 20). Because with this model, the heart is perfused with a buffer and therefore there are no leukocytes in the perfusate, the findings suggest that p38 inhibition has beneficial effects directly on the myocardium, in addition to its known effects on leukocyte recruitment and activation (Figure 3). This leukocyte-independent protective effect of p38 inhibition on the myocardium probably involves inhibition of I/R-induced production of cytotoxic cytokines by the heart and inhibition of p38-dependent proapoptotic pathways in cardiomyocytes. More recently, a newer-generation p38 inhibitor, SB239063[24] (Figure 1), that can readily be used in vivo has demonstrated beneficial effects in the intact rat model of I/R injury. In addition to direct protective effects of

p38 on cardiomyocyte survival, SB239063 produced a dramatic reduction in the myocardial inflammatory response, as evidenced by reduced upregulation of P-selectin and intercellular adhesion molecule and reduced neutrophil accumulation within the ischemic zone. Other related potential applications of p38 inhibitors include preservation of mechanical function of cold-stored hearts before transplantation.[25] This effect of p38 inhibition may be, in part, related to increased contractility caused by enhanced myofilament responsiveness to calcium.[26]

There are other potential applications for these cytokine-suppressive drugs, including the treatment of patients with heart failure. Although the RENEWAL and ATTACH trials,[27] targeting TNF-α by "capturing" it with a monoclonal antibody or a soluble receptor, produced negative results and raised concerns over worsening of heart failure, this of course does not necessarily mean that the concept of anticytokine therapies in heart failure is invalid, and it is conceivable that more broad-based anticytokine therapy, such as one achieves with p38 inhibitors, could be beneficial. Furthermore, we could benefit from the experiences of the oncologists that demonstrate that one may need to define the molecular phenotype or kinase activity profile of the individual patient, because just as with cancer, patients with the clinical diagnosis of "heart failure" are bound to have very different profiles (as evidenced by the lack of consensus on the signaling abnormalities present in the failing heart[17]). Although it is difficult, failing to do so may lead to discarding agents that are effective in subsets of patients. As an example, trastuzumab, the anti-HER2 tyrosine kinase receptor antibody, which confers a 22.5% improvement in overall survival in breast cancer patients with tumors that overexpress HER2 (25% to 30% of all breast cancers), would have been found to be of no value if it had been initially tested in breast cancer patients irrespective of the HER2 status.[6]

Other potential concerns with anticytokine therapies include a possible increased risk of infection, including reactivation of tuberculosis, and the development of opportunistic infections that have been seen with the anti-TNF therapies and with anakinra, an IL-1 receptor antagonist.[28] Of course, whether these issues are specific to the anti-TNF and anti–IL-1 therapies used or will be a general feature of all anticytokine therapies remains to be determined.

Stroke

Inhibition of several protein kinase pathways has been shown to be beneficial in animal models of stroke. These include the 3 families of MAPKs, the ERKs, JNKs, and p38 MAPKs.[14] In addition, cell culture studies suggest that inhibitors of glycogen synthase kinase-3 (GSK-3) may also be protective.[29,30] The first reports of neuroprotection in vivo with a kinase inhibitor used direct injection into the cerebral ventricles of PD98059 (Figures 1 and 2), a first-generation inhibitor of the activation of MEK1/2[9] (Figure 2 legend), the kinases that activate the ERKs.[31] This was followed by studies with intravenous administration of another MEK1/2 inhibitor, U0126 (currently in clinical trials for cancer; Table), which was also protective against forebrain and focal cerebral ischemia.[32] Remarkably, beneficial effects were seen with administration after 3 hours of ischemia, before reperfusion. These studies seemed counterintuitive, because the ERKs had generally been thought to be antiapoptotic in most settings (Figure 2), including in I/R injury in the heart.[19] The mechanism of protection may be prevention of excitotoxicity,[33] which is neuronal death caused by release of excitatory amino acids that activate metabotropic glutamate receptors. Excitotoxicity plays a critical role in I/R injury in the brain, and although the precise mechanisms of protection remain to be determined, MEK1/2 inhibitors may be blocking release of glutamate. In addition to stroke, MEK1/2 inhibitors have been reported to be protective against traumatic brain injury.[34] As one caveat, PD98059 and U0126 also block activation of MEK5,[9] the kinase that activates ERK5, the sole member of the fourth MAPK family. Thus, one cannot at this time formally rule out MEK5/ERK5 as the relevant target. Strikingly, another MEK1/2 inhibitor, PD184352, has been reasonably well tolerated when administered orally, twice daily, for 21 days (repeating every 4 weeks) in a phase I dose-ranging trial in cancer patients, with only fatigue, rash, and diarrhea being commonly reported.[6]

Inactivation of JNK3 (via gene deletion in a knockout mouse), which is selectively expressed in the central nervous system, and inhibition of p38 activation (by SB239063) were also protective in stroke models.[35,36] In the latter case, SB239063 reduced stroke-induced expression of TNF-α and IL-1β, cytokines that are believed to enhance neuronal loss after I/R. No fewer than 8 companies have reported the development of JNK inhibitors, many focusing on JNK3 and neuroprotection (stroke and neurodegenerative disorders).[37] Some have reported enhanced cell survival in a stroke model.[37] Safety studies with one agent (CC401, Table) are ongoing in healthy volunteers.[37]

Inhibitors of GSK-3 are being proposed as potential therapies for disorders as diverse as bipolar mood disorders (lithium and valproic acid are GSK-3 inhibitors), Alzheimer's disease (in which GSK-3 is believed to play a key role in formation of the neurofibrillary tangles and amyloid plaques, the latter being reduced by lithium in an animal model of Alzheimer's disease[38]), and stroke.[30] GSK-3 is inhibited when phosphorylated by the antiapoptotic kinase Akt, and at least part of the antiapoptotic effects of Akt are believed to be mediated by inhibition of GSK-3. GSK-3 inhibition may also mediate part of the phenomenon of ischemic preconditioning.[39] Published data are limited, but at this point, selective inhibitors (SB216763 and SB415286; Figure 1) have been shown to block neuronal cell death in culture induced by pharmacological inhibition of the PI3-kinase/Akt pathway or by polyglutamine toxicity caused by the Huntington's disease mutation.[29,40] Although promising, this kinase is a critical regulator of many basic cellular processes, including development, cardiac growth and hypertrophy, and tumorigenesis.[41,42] Therefore, it is likely that in the near future, inhibitors of GSK-3 will be restricted to relatively short-term use in high-risk patients.

Hypertension

Rho belongs to a family of small GTP-binding proteins that mediate intracellular signaling induced by activation of het-

erotrimeric G protein–coupled receptors and growth factor receptors. In the cardiovascular system, Rho regulates vascular smooth muscle contraction by modulating sensitivity to Ca^{2+}. One Rho effector is Rho kinase (ROCK), of which 2 isoforms have been identified. ROCKs phosphorylate the myosin-binding subunit of myosin light chain phosphatase and LIM kinase, ultimately regulating phosphorylation of myosin light chain and, via this mechanism, vascular smooth muscle cell contraction.[43] Therefore, it is tempting to speculate that ROCK inhibition could enhance coronary vasodilatation by changing Ca^{2+} sensitivity of coronary artery smooth muscle cells. In fact, a ROCK inhibitor, hydroxyfasudil, suppresses myosin light chain phosphorylation and significantly inhibits coronary spasm in a pig model. Two recent clinical trials of fasudil indicate that it may be an effective and well-tolerated antianginal agent[44] and also may be of benefit in patients with microvascular spasm of the coronary arteries.[45] Although the selectivity of fasudil against ROCKs is in question, these results suggest a potential use of ROCK inhibitors as novel agents to treat symptomatic patients with CAD. Another relatively specific ROCK inhibitor, Y-27632 (Figure 1),[9] is effective in lowering systolic blood pressure in spontaneously hypertensive rats, DOCA-salt rats, and renal hypertensive rats without affecting blood pressure in normal rats.[43] Collectively, selective ROCK inhibitors will probably be a novel approach to the treatment of hypertension. However, Y-27632 has also been shown to affect metastasis, neurite outgrowth, and contraction of smooth muscle cells other than vascular smooth muscle cells.[43] Therefore, the safety of Y-27632 and related agents remains a question and will need to be carefully evaluated in clinical trials.

Given the difficulty in controlling hypertension in elderly patients and diabetics, there will probably be many more targets against which inhibitors will be made. These could include the WNK (with no lysine) family of kinases, mutations of which are responsible for a rare hereditary form of hypertension, pseudohypoaldosteronism type II.[46] Because the WNK4 gene lies close to a locus showing the strongest linkage to blood pressure variation in the Framingham Heart Study, less severe mutations and polymorphisms of the WNK genes may play a more general role in hypertension. If so, these kinases might be ideal targets.

Diabetes and the Metabolic Syndrome

Another avenue open to manipulating activity of protein kinases is to identify drugs that activate, as opposed to inhibiting, a kinase. Protein kinases that are beneficially activated by allosteric mechanisms represent attractive targets for such therapies. One of these is the 5′-AMP-activated protein kinase (AMPK). AMPK exists in the cell as a heterotrimer of α, β, and γ subunits (the α subunit containing the kinase domain). Genetic mutations in the human $\gamma 2$ subunit of AMPK have been linked to hypertrophic cardiomyopathy and to ventricular preexcitation.[47] Specifically, these mutations are associated with a metabolic storage disorder marked by the accumulation of excess glycogen granules in the myocardium. Although the mechanisms by which these mutations lead to cardiomyopathy and preexcitation are not entirely clear, the mutations appear to inhibit activation of AMPK by AMP. Because AMPK inhibits glycogen synthase, the mutation could lead to increased glycogen synthase activity, increased glycogen production, and the observed accumulation of glycogen in the heart.

The reason that AMPK has generated a tremendous amount of interest on the part of pharmaceutical companies, however, is that activators of it could be useful in the treatment of patients with metabolic syndrome, diabetes, or hyperlipidemia.[48] AMPK was initially discovered in the early 1970s as an AMP-dependent kinase that inactivated HMG-CoA reductase and acetyl-CoA carboxylase (ACC).[49] It has since been established that AMPK functions as a cellular "fuel sensor" that is activated in times of reduced energy availability (when [AMP] is relatively high) and serves to inhibit anabolic processes (lipogenesis) and enhance glucose uptake.[49]

Several compelling lines of evidence point to the potential of AMPK as a useful drug target. ACC, the rate-limiting enzyme in fatty acid synthesis, catalyzes the formation of malonyl-CoA, a potent inhibitor of fatty acid oxidation. By inhibiting ACC, AMPK elevates fat oxidation.[49] In addition, AMPK activation leads to reduced levels of hepatic sterol response element–binding protein-1 and consequently suppresses the expression of several lipogenic genes. Thus, therapeutic activators of AMPK could reduce serum triglycerides. As an inhibitor of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, AMPK also functions to block cholesterol production,[49] and therapeutic AMPK activators could serve in a manner similar to the statins. In addition, AMPK is activated in exercise, triggering skeletal muscle glucose uptake in an insulin-independent manner. Of particular note, pharmacological activation of AMPK with 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) mimics exercise and triggers insulin-independent skeletal muscle glucose uptake. Thus, AMPK activators could also alleviate glucose intolerance. In support of this, the biguanide antidiabetic metformin may exert its effects in part by activating AMPK.[48]

The ability to activate AMPK in vitro with AMP and in vivo with AICAR (which is phosphorylated in the cell to ZMP, an analogue of AMP) and the observed antilipogenic and glucose transport effects of AICAR indicate that drugs targeting AMPK will need to be AMPK activators. It is likely that AMP-like compounds will provide the richest source of potential AMPK pharmaceuticals. Identification of such compounds will be assisted by the elucidation of the structural features of the AMPK AMP-binding pocket.

Conclusions

It is very likely that the next several years of translational cardiovascular research will feature a number of clinical trials using inhibitors of protein kinase signaling pathways to treat a variety of disorders. We have touched on some of the targets for which development of inhibitors is more advanced, but there are many others with great potential, including the β-adrenergic receptor kinase (heart failure)[50] and some kinase inhibitors that are currently in clinical trials for cancer and are in the discovery phase for atherosclerosis and restenosis (eg, growth factor receptors, including the platelet-derived growth

factor receptor, cell cycle regulators such as Cdk-1/-2, and protein kinase C) and for stroke (eg, Cdks).[51] Toxicity remains a major concern, because many of these kinases not only play roles in the pathogenesis of diseases but also function in pathways that regulate the most basic of normal cellular processes. That said, preclinical data have been reassuring. Toxicity data from clinical trials of these agents in cancer will be illustrative, but many of these studies have been designed to identify, or have used, the "maximum tolerated dose," which may be significantly higher than the doses that will be used in cardiovascular diseases. The use of combination therapy, targeting 2 or more kinases on the same or parallel pathways, may allow the use of lower (and therefore less toxic) doses and has shown some promise in cancer trials.[6] However, the majority of early trials will focus on individual kinases and their role in diseases for which only short-term therapy will be needed (eg, ACS or stroke) or for which targeted local delivery is possible. It must be realized, however, that these may not necessarily be the disease states most likely to benefit from therapy. Finally, as highlighted above, given the vast numbers of protein kinases in the human genome and their sequence and structural similarities, added to the inability to test the drugs against all kinases, specificity will remain a concern with these agents. Despite these obstacles, this new class of agents offers a great deal of promise to expand our therapeutic options for a wide variety of cardiovascular diseases.

Acknowledgments

This work was supported by US Public Health Service grants HL-61688 and HL-67371 to Dr Force, GM-46577 and DK-41513 to Dr Kyriakis, and a Basic Science Grant from the Arthritis Foundation to Dr Kyriakis. The authors thank Chris Vlahos for invaluable discussions. We sincerely apologize to those whose work could not be cited because of space limitations.

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TARGETING JNK FOR THERAPEUTIC BENEFIT: FROM JUNK TO GOLD?

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The c-Jun NH_2-terminal kinases (JNKs) phosphorylate and activate members of the activator protein-1 (AP-1) transcription factor family and other cellular factors implicated in regulating altered gene expression, cellular survival and proliferation in response to cytokines and growth factors, noxious stimuli and oncogenic transformation. Because these events are commonly associated with the pathogenesis of a number of human diseases, the potential of JNK inhibitors as therapeutics has attracted considerable interest. Here we discuss the evidence supporting the application of JNK inhibitors in inflammatory, vascular, neurodegenerative, metabolic and oncological diseases in humans, and describe the present status of drug discovery targeting JNK.

AP-1 TRANSCRIPTION FACTOR The transcription factor AP-1 is composed of homo- or hetero-dimers of proteins that belong to the FOS and JUN families. JUN proteins can homo-dimerize, but FOS proteins can only form stable dimers with JUN. AP-1 dimers can be phosphorylated by JNK and other MAP kinases and hence develop an enhanced DNA-binding capacity and transcriptional activity.

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doi:10.1038/nrd1132

JNK was initially identified and purified by Kyriakis *et al.* as a protein kinase that was activated in the liver of rodents exposed to cycloheximide[1]. Independent studies identified JNK as a stress-activated protein kinase that phosphorylated c-Jun on two sites in the NH_2-terminal activation domain[2,3]. Subsequent studies led to the molecular cloning of JNK[4,5] and the demonstration that it is a member of the mitogen-activated protein kinase (MAPK) group of signalling proteins[6]. Ten JNK isoforms are created by alternative splicing of messenger RNA transcripts derived from three genes: *JNK1*, *JNK2* and *JNK3* (FIG. 1)[7]. Gene disruption studies in mice demonstrate that JNK is essential for tumour-necrosis factor-α (TNF-α)-stimulated c-Jun phosphorylation and AP-1 TRANSCRIPTION FACTOR activity[8], and is also required for some forms of stress-induced apoptosis[9].

Certain cytokines, mitogens, osmotic stress and ultraviolet irradiation activate the JNK pathway, as depicted in FIG. 2. The upstream pathway leading to JNK activation is complex: cell- and stimulus-specific responses that lead to JNK activation are probably controlled by physically distinct intracellular complexes of multiple signalling proteins. JNK activation leads to the phosphorylation of a number of transcription factors — most notably the c-Jun component of AP-1 — and cellular proteins, particularly those associated with apoptosis (for example, Bcl2, p53 and so on).

JNK is activated by dual phosphorylation of the motif Thr-Pro-Tyr located in the activation loop[4]. JNK inactivation can be mediated by serine and tyrosine phosphatases, but also by a family of dual specificity MAP kinase phosphatases[6]. JNK phosphorylation is mediated by two MAPK kinases (MAPKKs) — MAP2K4 (also known as MKK4) and MAP2K7 (also known as MKK7) — that can cooperatively activate JNK. MAP2K4 preferentially phosphorylates JNK on tyrosine, whereas MAP2K7 preferentially phosphorylates JNK on threonine[10]. Gene disruption studies in mice demonstrate that both MAP2K4 and MAP2K7 are required for full activation of JNK by environmental stressors, and that MAP2K7 is essential for JNK activation by TNF[11]. The MAP2K4 and MAP2K7 protein kinases are also activated by dual phosphorylation within the activation loop, and this phosphorylation is mediated by one of a large group of upstream protein kinases, including transforming growth factor-β-activated kinase-1 and members of the MAPK/extracellular-regulated kinase group, the mixed lineage kinase group and the activator of S-phase kinase group of MAPKK kinases (MAPKKKs)[6]. Together, these protein kinases are able to form signalling cascades that can function as defined signalling modules that mediate JNK activation in response to specific stimuli (see FIG. 3).

The specificity of signal transduction by JNK is mediated, in part, by protein–protein interactions[12].

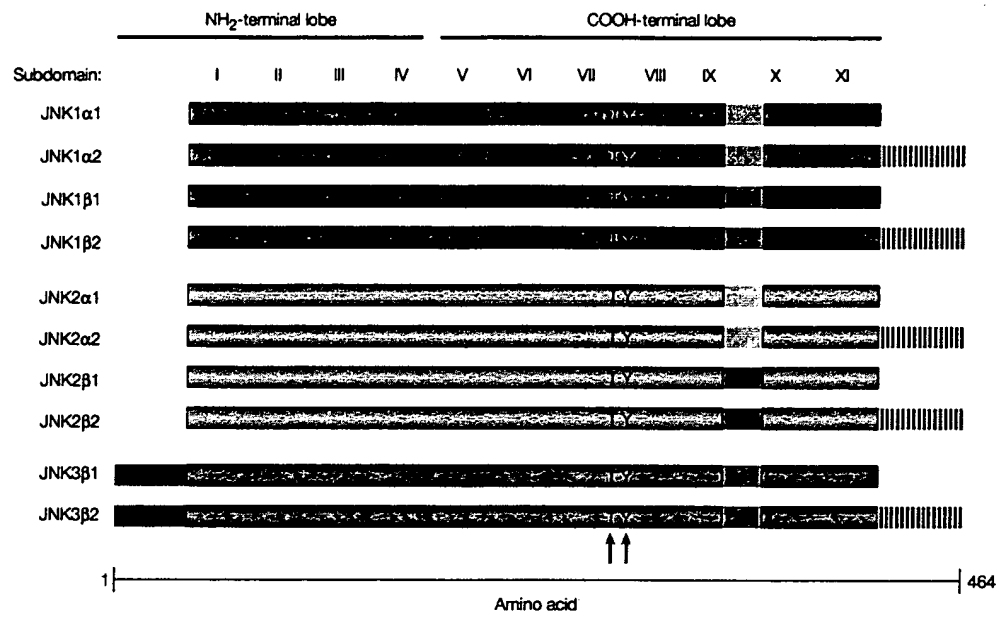

Figure 1 | **Structural features of the JNK proteins.** The c-Jun NH_2-terminal kinases (JNKs) are typical of serine/threonine kinases, comprising 11 protein kinase subdomains (indicated as I–XI). The protein kinase activation loop is located between domains VII and VIII, and contains the threonine (T) and tyrosine (Y) residues that are phosphorylated for full kinase activation. The members of the JNK family are generated by alternative splicing of three JNK genes (*JNK1*, *JNK2* and *JNK3*, depicted as orange, light and dark blue, respectively) to produce ten different isoforms. The differences are indicated by the shaded regions. There are two key alternative splicing sites: the first is between subdomain IX and X of the COOH-terminal lobe of the protein, which results in splice forms that demonstrate altered substrate specificity; the second alternative splicing site occurs at the C terminus of the protein, and results in proteins that differ in length by either 42 or 43 amino acids (depicted as hatched regions).

MAPKs contain a common docking site that is distant from the active site that binds to docking motifs (D-domain and FXFP) that are located in interacting proteins, including substrates, MAPKKs, and MAPK phosphatases[12]. JNK also interacts with scaffold proteins that can assemble functional signalling modules[13]. Examples of scaffold proteins include the JNK-interacting proteins (JIPs) that are transported by the microtubule motor protein kinesin[13]. Such scaffold proteins can regulate localized activation of JNK within cells.

The complexity of the JNK pathway provides multiple opportunities for the design of small-molecule inhibitors that might modulate signalling by the JNK pathway. Each target has both potential benefits and disadvantages for drug design. One approach is to directly target the JNKs, a strategy that is reviewed herein. This strategy is being aggressively pursued by a number of drug discovery companies, as evidenced by the patent and scientific literature. However, owing to the breadth of physiological functions mediated via signalling through the JNK family, direct inhibition at the level of the JNKs could also have liabilities. Alternatively, targeting the upstream MAPKKs or MAPKKKs within the ordered hierarchy of the signalling cascade could offer greater specificity in blocking pathological responses, and we refer the reader to excellent reviews of such agents[14,15].

Inflammatory diseases

Autoimmune and inflammatory diseases arise from inappropriate activation of the immune system, resulting in the overproduction of immune cells, inflammatory cytokines and tissue-destructive enzymes. These cells and proteins attack and destroy healthy tissue, giving rise to a number of diseases such as rheumatoid arthritis, multiple sclerosis, asthma, inflammatory bowel disease and psoriasis, as well as transplant rejection. Although available drugs alleviate many of the symptoms of disease, they generally do not target the underlying mechanisms, are relatively non-selective and have dose-limiting side effects. The search for agents that target the underlying pathogenic mechanisms of these diseases has accelerated during the past decade primarily due to our enhanced knowledge of molecular and genetic pathways regulating the immune system.

Activated immune cells express many genes encoding inflammatory molecules, including cytokines, growth factors, cell surface receptors, cell adhesion molecules and degradative enzymes. Many of these genes, including those encoding TNF-α, interleukin-2 (IL-2), E-selectin and matrix metalloproteinases (MMPs) such as collagenase-1, are regulated by the JNK pathway, through activation of the transcription factors AP-1 and ATF-2 (REF. 16).

Monocytes, tissue macrophages and tissue mast cells are key sources of TNF-α. The JNK pathway regulates

TNF-α production in bacterial lipopolysaccharide-stimulated macrophages, and in mast cells stimulated through the FcεRII receptor[17,18]. Inhibition of JNK activation effectively modulates TNF-α secretion from these cells. MMPs promote cartilage and bone erosion in rheumatoid arthritis, and generalized tissue destruction in other autoimmune diseases. Inducible expression of MMPs, including MMP3 and MMP9, and type II and IV collagenases, are regulated by activation of the JNK pathway and AP-1 (REF. 19). In human rheumatoid synoviocytes activated with TNF-α, IL-1 or Fas ligand, the JNK pathway is activated[20]. Inhibition of JNK activation results in decreased AP-1 activation and collagenase-1 expression. Activated JNK can also be detected in synovial fibroblasts and chondrocytes from the joints of osteoarthritic patients, but not from normal controls, and has been suggested to play a role in the chondrocyte injury and cartilage degeneration that are features of this disease[21,22]. The presence and activity of the JNK pathway in multiple cell types involved in the inflammatory process has drawn attention to the development of JNK inhibitors as immuno-modulatory agents (FIG. 4).

Recently, several studies have reported the effects of the administration of JNK inhibitors in animal models of arthritis and asthma. SP-600125 was reported as a selective inhibitor of JNKs 1, 2 and 3 (IC_{50} = 110–150 nM), but with much less activity against the closely related p38 MAPK (IC_{50} >30 μM)[23]. SP-600125 inhibits IL-1-induced phosphorylation of JNK and c-Jun in cultured synoviocytes from rheumatoid arthritis patients, and inhibits the production of MMP13, a key enzyme associated with cartilage destruction[21]. Administration of SP-600125 inhibits JNK activation and collagenase expression in the joints of rats with adjuvant arthritis. Animals showed a significant reduction in paw swelling and bone and cartilage damage. In light of these findings, the inhibition of JNK could be considered as a

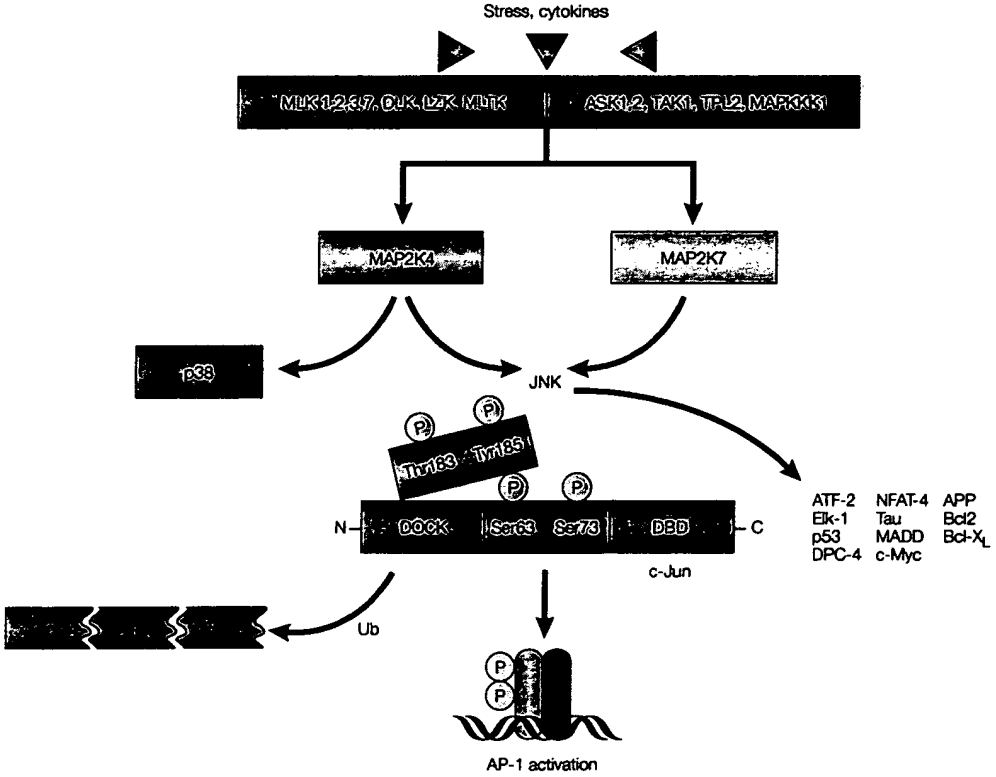

Figure 2 | **Organization of the JNK signal transduction cascade.** The c-Jun NH_2-terminal kinase (JNK) pathway is variably activated in cells by extracellular stimuli including stress and cytokines. A variety of receptor-associated signalling mechanisms lead to the activation of mitogen-activated protein kinase kinase kinases (MAPKKKs) that are capable of activating either MAP kinase kinase 4 or 7 (MAP2K4 or MAP2K7). MAP2K4 can activate either the JNKs or the p38-MAP kinases. MAP2K7 selectively activates the JNKs. JNK activation requires dual phosphorylation of both Thr183 and Tyr185, triggering the specific interaction of activated JNKs with a number of substrates including the c-Jun component of the activator protein-1 (AP-1) transcription factor. Resulting phosphorylation of c-Jun on Ser63 and Ser73 results in the acquisition of enhanced transcriptional activity of complexes containing AP-1. In the absence of serine phosphorylation, c-Jun is degraded by a ubiquitin (Ub)-dependent proteolytic pathway. APP, amyloid precursor protein; ASK, activator of S-phase kinase; ATF, activating transcription factor; Bcl, B-cell lymphoma protein; DBD, DNA-binding domain; DLK, dual leucine zipper kinase; DOCK, docking region; DPC-4, dystrophin-associated protein complex-4; Elk-1, member of the ETS oncogene family; LZK, leucine zipper-bearing kinase; MADD, MAPK-activating death domain; MLK, mixed lineage kinase; MLTK, mixed lineage kinase-related kinase; c-Myc, cellular myelocytomatosis oncogene; NFAT, nuclear factor of activated T cells; TAK, transforming growth factor-β-activated kinase; Tau, microtubule-associated protein tau; TPL2, tumour progression locus 2.

IC_{50}
The concentration of drug at which activity of a particular assay is inhibited by 50%. This is a typically used value to describe the relative potency of a drug agent.

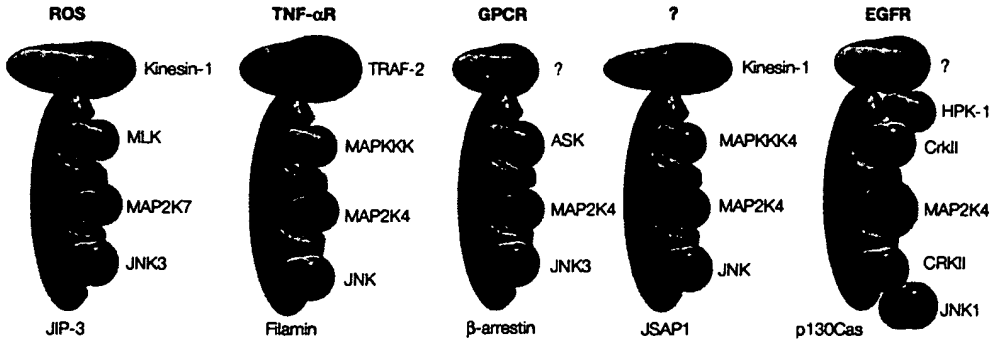

Figure 3 | **Modular organization of JNK signalling complexes.** The protein kinases that form c-Jun NH_2-terminal kinase (JNK) signalling modules in cells do so through interaction with scaffold proteins. Studies in yeast established the concept that scaffold proteins are crucial components of mitogen-activated protein kinase (MAPK) pathways. Five groups of potential scaffold proteins that might coordinate JNK signalling have been reported: JNK-interacting protein (JIP), filamin, β-arrestin, JNK/stress-activated protein kinase-associated protein 1 (JSAP1) and p130Cas. These scaffold proteins bind discrete members of the JNK pathway, and co-localize them within the cell. It seems that these scaffolds provide spatial and stimulus-specific regulation of JNK function. JIP belongs to a group of proteins that were identified as proteins that bind JNK, but were subsequently shown to bind other components of the pathway, including mixed lineage kinase (MLK) and MAPK kinase 7 (MAP2K7). Filamin is a large protein that interacts with and organizes actin filaments, but also binds to MAPKK kinase (MAPKKK), MAP2K4 and JNK. The arrestin group of adapter proteins, including β-arrestin 2, bind to G-protein coupled receptors (GPCRs) following ligand engagement and have important functions in the termination of heterotrimeric G protein activation by GPCRs. Recent studies demonstrate that β-arrestin 2 can bind components of the JNK pathway. p130Cas is a multiprotein complex that includes JNK, recruited through association with v-crk sarcoma virus CT10 oncogene (CRKII). A more detailed review of JNK complexes is presented in REF. 103. ASK, activator of S-phase kinase; EGFR, epidermal growth factor receptor; HPK-1, haematopoietic progenitor kinase-1; ROS, reactive oxygen species; TNF-αR, tumour-necrosis factor-α receptor; TRAF, TNF receptor-associated factor.

potential therapy for rheumatoid arthritis. These studies were extended using JNK2 knockout mice in a model of passive murine collagen-induced arthritis, in which JNK2 was demonstrated to be a key determinant of matrix degradation, but was less important for inflammation and paw swelling[24]. These data indicate that optimal treatment for rheumatoid arthritis might require combined JNK1 and 2 inhibition. The JNK inhibitor SP-600125 also reduces bronchoalveolar accumulation of eosinophils and lymphocytes in animals subjected to repeated allergen exposure, and reduces serum Immunoglobulin E levels, indicating its possible use in the treatment of asthma[25].

Inappropriate activation of T lymphocytes initiates and perpetuates many autoimmune diseases, including asthma, inflammatory bowel disease and multiple sclerosis. Studies of immature T cells (thymocytes) have not demonstrated a role for JNK in early development. However, CD3-mediated apoptosis of $CD4^+$ $CD8^+$ double-positive thymocytes caused by the administration of a monoclonal antibody to CD3 *in vivo* is reduced in *JnK*-null mice[26,27]. This observation indicates that JNK might contribute to negative selection of autoreactive T cells in the thymus, but a direct test of this hypothesis has not yet been carried out. Exposure of $CD4^+$ T cells to antigen causes these cells to differentiate into effector T_H1 or T_H2 cells, which secrete cytokines that control the type of immune response that is generated. T_H1 cells promote cell-mediated immunity against intracellular microbial pathogens by expressing interferon-γ (IFN-γ), IL-2 and lymphotoxin. By contrast, T_H2 cells express cytokines (IL-4, IL-5, IL-9, IL-10 and IL-13) that promote humoral immunity against parasites and extracellular pathogens. Studies of compound mutations of *JnK1* and *JnK2* demonstrate that JNK is not required for $CD4^+$ T cell activation, but that JNK is required for differentiation to effector cells[28]. So, *JnK1*-null $CD4^+$ T cells selectively differentiate into T_H2 effector cells[29]. Similarly, *JnK2*-null $CD4^+$ T cells produce less IFN-γ during differentiation and, as a result, express low levels of the β2 subunit of the IL-12 receptor and fail to differentiate into the T_H1 subtype[30].

$CD8^+$ T cells can differentiate into cytotoxic T cells that help defend the host during cell-mediated immune responses by secretion of IFN-γ, TNF-α, perforin and granzyme. Interestingly, JNK1 and JNK2 seem to have different roles in this response[31,32]. Studies of *JnK1*-null mice demonstrate that antigen-driven $CD8^+$ T-cell expansion is severely reduced *in vivo* and *in vitro*. This defect is associated with reduced AP-1 transcription activity and reduced cytokine expression (IL-2 and IFN-γ) and is caused, at least in part, by failure of the $CD8^+$ cells to express CD25, the α chain of the IL-2 receptor. By contrast, *JnK2*-null $CD8^+$ T cells express greatly increased amounts of IL-2 and IFN-γ. Together, these data indicate that JNK1 and JNK2 have positive and negative roles, respectively, in the $CD8^+$ T-cell immune response. These different roles of JNK1 and JNK2 indicate that the effect of pharmacological inhibition of JNK in $CD8^+$ T cells could be complex. Nevertheless, studies using the small-molecule JNK inhibitor SP-600125 demonstrate that this drug mimics JNK1 deficiency by inhibiting antigen-driven $CD8^+$ T-cell expansion and severely reduces the expression of IL-2

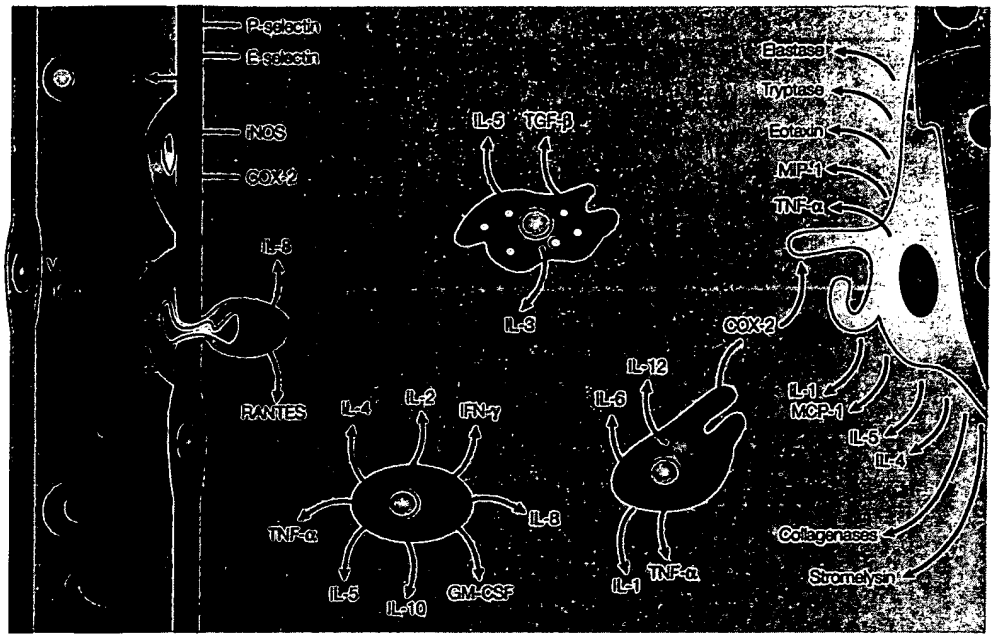

Figure 4 | **Inflammatory gene expression and JNKs.** Many autoimmune diseases originate from an imbalance in normal immune responses to tissue injury, infection or immune surveillance. In response to these stimuli, vascular endothelium surrounding tissues is activated, resulting in increased expression of endothelial cell adhesion molecules such as P- and E-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and chemotactic cytokines such as interleukin-8 (IL-8) or RANTES. These molecules promote the margination, activation and extravasation of blood leukocytes. These leukocytes, including T lymphocytes, macrophages and neutrophils, are activated during this process and express distinct sets of secreted products that contribute to the resolution of tissue trauma or infection. Local cells, including resident mast cells or tissue macrophages, and epithelial and fibroblast-like cells, are also activated and express discrete sets of products. Under normal homeostatic conditions, an inflammatory response will resolve itself. However, in autoimmune disease, the inflammatory response is not resolved, with chronic inflammation leading to significant tissue destruction and remodelling. Many of the gene products expressed by the activated cells involved in the inflammatory response are regulated by the transcription factor activator protein-1 (AP-1), and the c-Jun NH_2-terminal kinase (JNK) pathway. COX-2, cyclooxygenase-2; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LFA-1, lymphocyte function associated protein-1; Mac-1, macrophage antigen α-polypeptide; MCP-1, membrane cofactor protein-1; MIP-1, major intrinsic protein-1; TGF-β, transforming growth factor-β; TNF-α, tumour-necrosis factor-α; VLA-4, integrin α4.

and IFN-γ[32]. This observation is consistent with the results of biochemical measurements of JNK activity that indicate the presence of high levels of JNK1 activity and low levels of JNK2 activity in activated $CD8^+$ T cells.

The JNK pathway is activated in T cells by antigen stimulation and CD28 receptor co-stimulation, and regulates production of the growth factor IL-2 and cellular proliferation[33]. T cells activated by antigen receptor stimulation in the absence of accessory cell-derived co-stimulatory signals lose the capacity to synthesize IL-2, a state called clonal anergy. This is an important process by which autoreactive T-cell populations are eliminated from the peripheral circulation. Of note is that anergic T cells fail to activate the JNK pathway in response to CD3- and CD28-receptor co-stimulation, even though expression of the JNK enzymes is unchanged[34].

In summary, JNKs seems to play multiple roles in T-cell immune responses[35]. JNK inhibition does not block $CD4^+$ T-cell activation, but does selectively inhibit T_H1-mediated immune responses. By contrast, JNK inhibition does inhibit $CD8^+$ T-cell activation. These data indicate that JNK is a potential therapeutic target that might allow the selective modulation of effector T cell function in diseases such as rheumatoid arthritis, asthma and chronic transplant rejection.

NEUROFIBRILLARY TANGLES Intracellular aggregates of paired helical filaments composed primarily of hyper-phosphorylated Tau proteins. Tau is a microtubule-associated protein found within neurons and normally restricted to axons. Hyper-phosphorylated Tau forms tangled masses that consume the neuronal cell body, presumably leading to neuronal dysfunction and ultimately cell death.

Neurodegenerative diseases

Neurodegenerative diseases, including Alzheimer's, Parkinson's and Huntington's diseases and stroke, share synaptic loss, neuronal atrophy and death as common pathological hallmarks. During the past decade, pharmaceutical research on these diseases has shifted focus from symptomatic benefit to developing novel disease-modifying agents. A key driver of this focus is the enhancement in fundamental knowledge of the mechanisms governing neuronal survival and death. JNK plays an integral role in neuronal death and this pathway might be operative in various central nervous system (CNS) disease states (FIG. 5).

JNKs 1 and 2 exhibit a broad tissue distribution, whereas JNK3 is predominantly localized to brain and testes. In the human CNS, the major JNK isoforms expressed are JNK3α1 and JNK1α1 (REF. 36), with JNK3 preferentially localized to pyramidal neurons in the CA1

and CA2 regions of the hippocampus and in specific subregions of the neocortex.

Knockout mice lacking either *JnK1*, *JnK2* or *JnK3* develop normally[36,37]. However, mice lacking both JNKs 1 and 2 die prematurely and exhibit brain abnormalities that are attributable to a dysregulation of apoptosis. So, JNK1 and JNK2 might be redundant in function for embryonic brain development. Although, disruption of *JnK3* in developing mice is of no apparent consequence, a pathological role for JNK3 downstream of stress-inducing stimuli is evident. In cell culture, increased expression of c-Jun, c-Jun phosphorylation and/or JNK activity correlates with neuronal apoptosis induced by a variety of stimuli[38,39]. JNK3 deficiency blocked c-Jun phosphorylation, c-Jun induction and sympathetic neuron death following trophic factor withdrawal[40].

Alzheimer's disease is characterized by progressive memory loss and deterioration of cognitive function. Pathologically, the hallmarks of Alzheimer's disease include a prevalence of amyloid deposits, NEUROFIBRILLARY TANGLES (NFTs) and neuronal synapse and cell loss, predominantly in the cortex and hippocampus. Genetic analyses of familial Alzheimer's disease cases provided links to mutations in the amyloid precursor protein (APP) and the presenilin genes *PSEN1* and *PSEN2*, which might therefore have a role in Alzheimer's disease pathogenesis. Extending these findings, animal models revealed that the identified mutations in the APP and the *PSEN1* and *PSEN2* genes affect the generation and deposition of β-amyloid fragments[41]. Direct toxicity of β-amyloid is postulated to contribute to the neuronal dysfunction and loss observed in Alzheimer's disease. *In vitro* exposure of neurons to β-amyloid fragments results in neurite atrophy and cell death with morphological and biochemical characteristics consistent with an apoptotic process. β-Amyloid-induced cell death is attenuated in cortical neurons from *JnK3*-null mice, and JNK3 mediates this cell death through the activation of c-Jun and the enhanced expression of Fas ligand[42].

Post-mortem brain sections from Alzheimer's disease patients revealed an altered distribution and activation of JNKs. JNKs 1, 2 and 3 distributed to different subcellular structures specific to the Alzheimer's disease brain[43,44], thereby indicating either a causal role in, or a response to, the pathology. JNK phosphorylates Tau *in vitro* at sites identified in paired helical filament Tau, the major constituent of NFTs[45]. Phospho-JNK staining was also localized in transgenic mice overexpressing a mutant form of PSEN1 to neurons surrounding amyloid plaques, as well as to neurons that contained intracellular accumulations of β-amyloid[46].

A direct role for the JNK pathway in functional regulation and metabolism of APP has also been postulated. APP is a substrate for JNK3 (REF. 47). The JNK phosphorylation site of APP is Thr668, a site that is also phosphorylated by MAPK3 (also known as extracellular-regulated kinase-1 (ERK1)), glycogen synthase kinase 3β (GSK3β), and CDK5/p35 (although each of these kinases phosphorylates APP to a lesser extent than JNK). Clearly, signalling through the JNK pathway is relevant to multiple physiological and pathological events that might be operative in Alzheimer's disease.

Parkinson's disease is characterized by behavioural impairments resulting from the relatively selective death of dopaminergic neurons. Similarly to Alzheimer's disease, the neuronal loss in Parkinson's disease is progressive and occurs over an extended timeframe. Several studies examining Parkinson's disease autopsy brains revealed that apoptosis could be the underlying mode of death of the vulnerable neurons[48]. So far, there is no direct evidence of JNK activation in Parkinson's disease autopsy brains, so its involvement in the pathological process is inferred from the results of cell culture and animal model studies.

The best characterized and most relevant animal models of Parkinson's disease use the selective nigrostriatal dopaminergic neurotoxin 1-methyl-4-phenyl-tetrahydropyridine (MPTP). MPTP administration to experimental animals produces a pattern of neurodegeneration and a neuropathology that is nearly identical to that seen in the brains of post-mortem human Parkinson's disease specimens. Recently, adenoviral gene transfer of the JNK-binding domain of JIP-1, which acts as an inhibitor of JNK function, into the striatum of mice inhibited MPTP-mediated activation of JNK, c-Jun and caspase in the substantia nigra. This treatment also blocked neuronal death in the substantia nigra and the loss of catecholamines in dopaminergic terminals[49]. This agent attenuated behavioural impairment as measured by amphetamine-induced locomotor increases.

Ischaemic injury to the CNS can lead to neuronal injury and death through a number of mechanisms. Several factors have been defined that mediate cell death after ischaemia, including excitotoxicity, elevated intracellular calcium levels, inflammatory processes mediated by cytokines and loss of survival factors[50]. In a model of transient focal ischaemia, elevated levels of phospho-c-Jun co-localized with TUNEL-labelled neurons in the cortex, which indicates the activation of upstream JNKs[51]. The co-localization with TUNEL-labelled neurons indicates a causative role for JNK activation and apoptosis.

Mice deficient in JNK3 or expressing mutations in the phosphorylation site of c-Jun are resistant to the hippocampal neurotoxic events associated with administration of the glutamate receptor agonist kainic acid. Mice that are deficient in JNK1 or JNK2 are not resistant to either kainic acid-mediated seizures or neuronal death[37]. These data indicate that the different JNK isoforms regulate differential responses to neuronal insults; in particular, the JNK3 isoform is involved in glutamate excitotoxicity, an important component in ischaemic death, and is expressed in a brain region that is vulnerable to global ischaemic conditions.

Metabolic disease

Obesity and type 2 diabetes are the most prevalent and serious of the metabolic diseases[52]. Insulin resistance is closely associated with these syndromes, and is commonly evident in hypertension, and following infection and injury. In these settings, β-cells within the pancreas

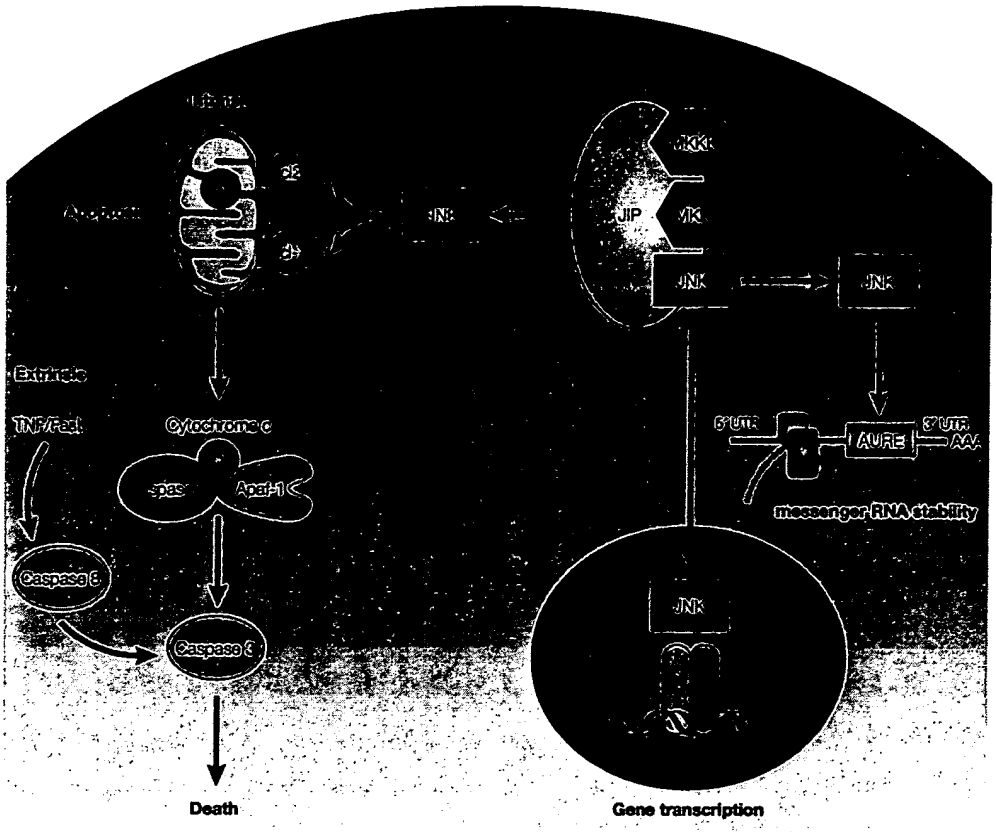

Figure 5 | **Biological functions of JNK.** C-Jun N-terminal kinases (JNKs) regulate cell survival and apoptosis by distinct mechanisms. JNKs play a key role in regulating the transcription and translation of cellular genes involved in the stress response. Activated JNKs interact with activator protein-1 (AP-1) and other transcription factors to modulate transcription of a number of genes. JNKs can also modulate the half-life of a set of genes that contain AU-rich elements (AURE) in their 3' untranslated regions (UTR), an element associated with rapid turnover and short half-life. Activation of JNKs results in a repression of turnover via these elements, thereby enhancing translation of these messenger RNAs. Activated JNKs can also promote cellular apoptosis by activating an intrinsic pathway whereby Bcl2 (B-cell lymphomas 2) and BCL-xL promote release of pro-apoptotic molecules including cytochrome c from mitochondria. This leads to the activation of caspases and cell death. JIP, JNK-interacting protein; MKK, mitogen-activated protein (MAP) kinase kinase; MKKK, MAP kinase kinase; TNF, tumour-necrosis factor.

fail to secrete sufficient insulin to compensate for peripheral insulin resistance. Insulin resistance and compensatory hyper-insulinaemia dysregulate many physiological processes that contribute to life-threatening metabolic, vascular and cardiac diseases[53]. Although new drugs are emerging to improve insulin sensitivity, the molecular mechanisms of insulin resistance have been the subject of intensive research. The idea that inflammation causes insulin resistance has been held for some time and is consistent with the concept that anti-inflammatory drugs, such as high dose aspirin, promote insulin sensitivity. The physiological response to infection, physical or thermal injury, or obesity invariably involves the production of pro-inflammatory cytokines, such as TNF-α, that activate various serine kinases. Considerable evidence indicates that serine phosphorylation of the insulin receptor or the insulin receptor substrate (IRS) proteins might inhibit insulin signalling and promote insulin resistance.

During obesity, adipocytes produce TNF-α, which promotes insulin resistance and stimulates serine phosphorylation of IRS1, whereas disruption of TNF receptor-1 partially restores insulin signalling and glucose tolerance in obese mice[54]. Insulin signalling complexes are assembled by insulin-stimulated tyrosine phosphorylation of scaffold proteins, including the IRS proteins, Src homology 2 domain-containing transforming protein, adaptor protein with pleckstrin homology and src homology 2 domains, GABA (γ-aminobutyric acid) receptors 1/2, Cas-Br-M (murine) ecotropic retroviral transforming sequence. Though the role of each of these components is of interest, transgenic mice studies revealed the importance of Irs1 and Irs2 for somatic growth and carbohydrate metabolism[55,56]. More than 100 potential serine phosphorylation sites exist in Irs1, and many protein kinases phosphorylate Irs1 *in vitro*, including JNK. The Irs proteins contain a binding site for the docking

domain of JNK. Activation of JNK by pro-inflammatory cytokines inhibits insulin signalling in mouse embryonic fibroblasts, 3T3-L1 and 32Dir cells through phosphorylation of Ser307 of Irs1. Insulin-stimulated Irs1 Ser307 phosphorylation was inhibited by almost 80% in cells lacking JNK1 or JNK2, or in cells expressing a mutant Irs1 protein lacking the JNK-binding domain[57]. Insulin activates JNK activity in other cell types, including L6 monocytes, rat adipocytes and Rat-1 fibroblasts, indicating that activated JNK could be an important negative feedback regulator of insulin signalling[58].

Striking evidence for a role of JNK in insulin resistance and obesity came from the finding that mice fed on a high-fat diet, and *ob/ob* mice that are genetically prone to obesity, exhibit spontaneously high JNK1 activity in liver, skeletal muscle and fat. Mice lacking JNK1 show decreased adiposity, significantly improved insulin sensitivity and enhanced insulin receptor signalling in the high-fat and *ob/ob* models[59]. As a mediator of obesity and insulin resistance, as well as many other cellular processes including apoptosis and neural differentiation, JNK is a potential therapeutic target for obesity and type 2 diabetes.

Cancer

The causal routes by which signal transduction pathways contribute to cellular transformation and tumorigenesis are well established. A substantial body of evidence indicates that JNK activation and c-Jun phosphorylation are required for transformation induced by *RAS*, an oncogene that is mutationally activated in almost 30% of human cancers[60]. Ras induces phosphorylation of c-Jun on the same serine residues phosphorylated by JNK[4,61], and acts cooperatively with c-Jun to enhance cellular transformation[62]. Fibroblasts lacking c-Jun cannot be transformed by Ras, which indicates a requirement for c-Jun in this process[63]. Moreover, recent studies have demonstrated the requirement of c-Jun for the development of chemically induced liver tumours in a model of hepatocellular carcinoma[64]. One of the functions of c-Jun that might contribute to tumour development is the transcriptional repression of the gene that encodes the p53 tumour suppressor[65]. Taken together, these data strongly support a role for c-Jun in cellular transformation.

JNK also seems to play a significant role in tumour development. Several tumour cell lines have been reported to possess constitutively active JNK[66]. The transforming potential of several oncogenes is reduced after the introduction of antisense JNK oligonucleotides or dominant-negative versions of proteins belonging to the JNK pathway[67,68]. A series of transfection studies demonstrated that the sites of c-Jun phosphorylation by JNK are required for efficient co-transformation activity with activated Ras[61]. Moreover, fibroblasts from mice harbouring a mutated *c-Jun* allele that lacks the JNK phosphorylation sites (JunAA) are resistant to transformation induced by activated Ras and Fos[69]. c-Fos-induced osteosarcomas and skin tumours induced by chronic activation of the Ras pathway are reduced in JunAA mice. Collectively, these data indicate that JNK activity is necessary for efficient transformation and tumorigenesis by oncogenes such as *Ras*.

However, in a recent study, fibroblasts isolated from mice that lack expression of JNK due to compound mutations of the *JnK* genes were efficiently transformed by Ras, and actually formed increased numbers of tumour nodules and size of individual tumours in mice injected with these cells[70]. This enhanced tumour formation seemed to be due to the absence of JNK-stimulated apoptosis in Ras-induced *JnK*-null tumours. An important aspect of tumour development is the suppression of apoptosis, and human tumours seem to utilize several different mechanisms to evade cell suicide, including enhanced expression of B-cell leukaemia/lymphoma 2 (Bcl2), p53 and apoptotic protease activating factor-1. So, a normal function of JNK might be to suppress tumour formation by activating apoptosis. How might this be mediated? Examination of *Jnk*-null primary fibroblasts has shown that JNK is necessary for stress-induced mitochondrial release of proapoptotic molecules, including cytochrome *c* (see FIG. 3)[9]. Activated JNK is sufficient to cause caspase-independent release of cytochrome *c* and subsequent apoptosis. Studies of fibroblasts have revealed a requirement for both Bax and Bak — pro-apoptotic members of the Bcl2 family — in JNK-mediated cytochrome *c* release and apoptosis[71]. Together, these data indicate that JNK activates apoptosis by interactions with the Bcl2 family of proteins. Therefore, it is possible that JNK might promote or suppress tumour development in different settings.

It is probable that JNK1 and JNK2 are not tumour suppressors, because they are ubiquitously expressed and exhibit a strong degree of functional redundancy, and the likelihood of mutational loss of both *JNK* genes is extremely low. However, JNK3 expression is largely restricted to the brain and has functions that are not compensated for by JNK1 or JNK2. Indeed, since mutations in *JNK3* were identified in ten out of nineteen human brain tumours examined[72], *JNK3* can be considered a candidate tumour suppressor gene. Similarly, the upstream activators of JNK (MAP2K4 and MAP2K7) serve nonredundant roles and so could also be tumour suppressor genes[73,74]. It is interesting in this regard that mutations in *MAP2K4* have been identified in human cancers of the pancreas, lung, breast, colon and prostate[75,76]. Interestingly, previous studies have identified *MAP2K4* as a metastasis suppressor gene. Loss-of-function mutations in *MAP2K4* are associated with aggressive growth and metastasis of prostate and ovarian cancers[77,78]. These data are consistent with the recently published study of *JNK*-null fibroblasts that indicate a role for JNK in metastasis suppression[70]. However, a direct test of this hypothesis in a reliable animal model of metastasis will be required to confirm whether JNK plays a role in metastasis suppression, or in tumour surveillance.

Although JNK seems to act as a tumour suppressor in fibroblast transformation caused by oncogenic Ras, JNK can contribute to proliferation or survival of other tumour cell types. JNK can potentiate B-cell lymphoma

caused by breakpoint cluster region-Abelson murine leukaemia oncogene, because JNK is required for tumour cell survival[79]. Studies of various cancer cell lines have revealed high levels of JNK activity[66], and inhibition of JNK using antisense approaches can reduce oncogenic transformation in some tumour cell lines[67,68].

Together, these considerations indicate that JNK could play more than one role in tumour development, and that in certain cases this role might be to promote or inhibit tumour development. Gaining a deeper understanding of the genetic and mechanistic basis for these different roles of JNK in tumours is essential for determining the true potential of JNK inhibitors as anticancer therapeutics.

JNK drug discovery

During the past decade, a combination of high-throughput screening, kinase-specific libraries and structure-based drug design has facilitated the discovery of selective kinase inhibitors. Screening of natural products of different origin, and collections of available compounds, has led to the identification of compounds that served as templates for medicinal chemistry efforts to design selective inhibitors for a range of kinase targets[80]. This information has facilitated the design, synthesis and screening of libraries of compounds that have structural features of inhibitors that interact at the kinase ATP site[81]. Determination of the X-ray structure of the members of the MAPK family, ERK[82], p38 (REF. 83) and JNK3 (REF. 84) has revealed approaches for the design of potent, yet selective, inhibitors of the JNKs. These efforts have led to the patenting of a series of JNK inhibitors, as described below.

Signal Pharmaceuticals (now Celgene) reported the discovery of a series of pyrazoloanthrone derivatives of compound 1 (SP-600125) as inhibitors of the JNK pathway for the treatment of autoimmune, anti-inflammatory and neurodegenerative diseases[23,85]. Compound 1 (FIG. 6) showed IC_{50} values of 110 nM for JNK1 and JNK2, and 150 nM for JNK3. Compound 1 was also evaluated for selectivity against a number of kinases and showed greater than 30 μM inhibitory activity against p38-2, ERK1, MAPKKK1, IKK1, inhibitor of κ kinase-β (IKK2), protein kinase A, protein kinase C and epidermal growth factor receptor. Recently, SP-600125 was tested under different conditions against a broader range of kinases, in which it inhibited several other kinases with a similar or greater potency than the JNKs[86]. The true selectivity of this compound has yet to be resolved, but it does seem to be a valuable tool for assessing the role of JNK in various disease models. In cellular assays, SP-600125 inhibited TNF-α production in monocytes and IL-2 production in Jurkat cells with an IC_{50} of approximately 5 μM. In rats, compound 3, administered 15 minutes before lipopolysaccharide, blocked TNF-α production when dosed intravenously and orally. SP-600125 inhibited leukocyte recruitment in a rat model of allergic airway inflammation at 30 mg per kg subcutaneously[25], and blocked JNK activation, MMP3 expression, and joint destruction in a rat adjuvant arthritis model[21]. In the rat, SP-600125 also blocked kainic acid-induced seizure activity by approximately 30%.

A second series of JNK-selective inhibitors (compound 2) were also recently disclosed from Celgene[87]. Celgene initiated a single, escalating-dose Phase I safety trial in normal healthy volunteers late in 2002 (REF. 88). The identity of the JNK inhibitor compound under investigation is unknown at this time.

A series of pyrimidinyl-substituted benzazole-acetonitriles (compound 3; FIG. 6) designed by Serono were disclosed as inhibitors of JNK2 and JNK3 for the treatment of autoimmune and neuronal diseases[89]. The benzazoles are more potent inhibitors of JNK3 than JNK2, with several compounds inhibiting JNK3 in the 30–70 nM range. Serono also disclosed a large series of sulfonyl amino acid[90], sulfonamide[91] and sulfonyl hydrazides[92] (compound 4) as inhibitors of JNK2 and JNK3. The inhibitors were reported to promote survival of sympathetic neurons in culture and to protect against cell death during stroke following global ischaemia in gerbils. It is not known whether these inhibitors are competitive with the ATP site, with the substrate site, or are producing inhibition in a noncompetitive manner.

Vertex reported a series of 3-oximido-oxindole analogues (compound 5) for the treatment of stroke and neurodegenerative diseases[93]. A number of compounds containing benzo-1,3-dioxolane groups inhibited JNK3 with IC_{50} values less than 100 nM. Vertex also disclosed a series of 4-substituted isoxazole analogues (compound 6) containing a 2-anilinopyridine or 2-anilinopyrimidines as JNK3 inhibitors[94]. A variety of substituents were tolerated on the aromatic rings, resulting in potent JNK3 inhibitors.

Researchers at Takeda disclosed the preparation of azoles as JNK inhibitors (compound 7)[95]. These compounds showed *in vitro* IC_{50} values of 30–210 nM against JNK1. In an *in vitro* assay using THP-1 cells, compounds of this class inhibited TNF-α production with IC_{50} values of 2–100 nM.

Hoffman-LaRoche reported a series of 4-aryl- (compound 10) and 4-alkynyl- (compounds 11–13) isoindolones (compound 8) as inhibitors of the JNKs[96,97]. Several alkynyl analogues inhibited JNK/SAPK with IC_{50} values less than 150 nM.

Hoffman-LaRoche also disclosed a series of 4,5-pyridazinoxindole JNK inhibitors (compound 9) for the treatment of neurodegenerative and inflammatory diseases[98]. A variety of substitutions were tolerated on the pyridazine moiety. A derivative in which R1 and R2 are simple alkyl or fused cycloalkyl groups, such as cyclohexyl 17, exhibited IC_{50} values below 100 nM for JNKs.

Recently, researchers at Aventis claimed new substituted indolizine derivatives (exemplified by compound 10) as JNK inhibitors useful for the treatment of cancer, asthma and arthritic diseases[99]. No pharmacological data for these compounds has been reported.

A series of 1H-indazole derivatives of compound 11 from Eisai were reported as JNK inhibitors with

IC_{50} values of about 50 nM against JNK3, and as being useful for the treatment and prevention of Alzheimer's and Parkinson's diseases[100]. Additionally, imidazole derivatives of compound 11 were claimed to be potent JNK3 inhibitors with IC_{50} values of 6 nM against JNK3 (REF. 101).

Researchers at Merck claimed the use of 4-(4-pyrimidinyl)-5-phenylimidazole derivatives as JNK inhibitors[102]. Compound 12 was reported to inhibit JNK3α1 *in vitro* with an IC_{50} of 1 nM. These compounds were claimed to have utility as apoptosis inhibitors for the treatment and prevention of stroke, Parkinson's disease,

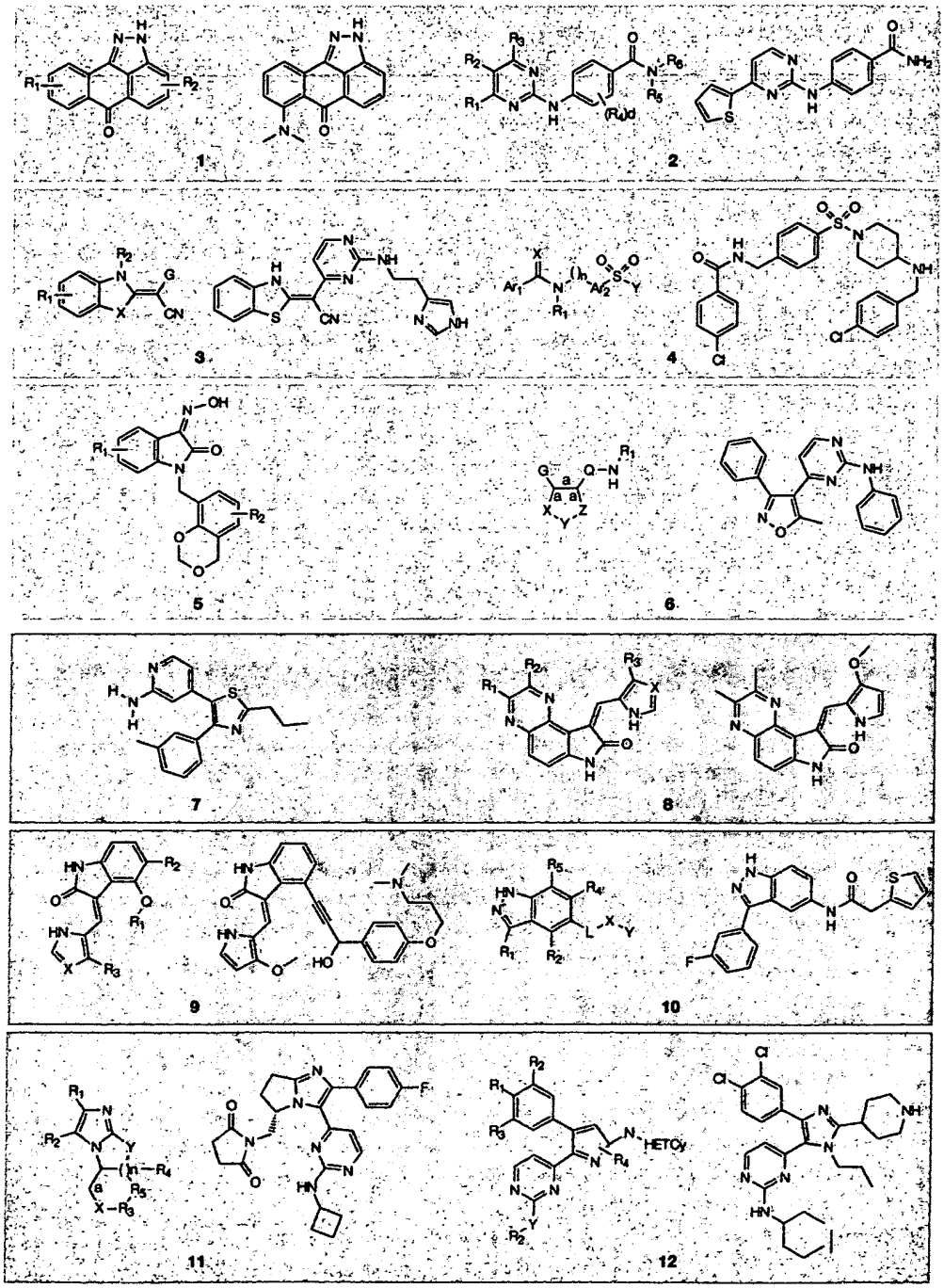

Figure 6 | **Inhibitors of JNKs reported in the patent literature.** In most cases, the generic core structure is represented on the left, with specific examples cited on the right. JNK, c-Jun NH_2-terminal kinase.

Alzheimer's disease, amylotrophic lateral sclerosis, multiple sclerosis, spinal cord injury, head trauma and seizures. No pharmacological data have been disclosed.

Conclusions

Data continues to emerge implicating the JNK pathway in a number of physiological and pathological functions that are probably operative in a range of human diseases. The shear breadth of the diseases in which JNK inhibitors could show benefit has attracted many pharmaceutical companies seeking blockbuster opportunities and maximal return on their research investment. Our understanding of the organization and function of all levels within the JNK signalling cascade continues to evolve. Because of the complex cross-talk within this signalling cascade, as well as its cell-type- and response-specific modulation, it is difficult to predict potential adverse events that might arise from pathway inhibition. The fact that compounds that inhibit the JNK pathway are progressing in clinical trials bears hope that sufficient safety and risk–benefit margins will be observed. In the coming years, the utility of targeting this pathway for therapeutic benefit will probably be determined.

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Acknowledgements
Special thanks to A. Lewis and B. Bennett at the Signal Research Division of Celgene for permission to reproduce FIGS 3 and 5.

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